The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Preparation of Aldehyde-Fuchsin from Aqueous Solution of Basic Fuchsin

By

Tom Mori, Sei Kato and **Noriko Fujimori**
(森　　富) (加 藤　聖)　　(藤 盛 則 子)

From the Laboratory of Assist. Prof. T. Mori, Department of Anatomy, Tohoku University School of Medicine, Sendai

(Received for publication, February 15, 1958)

Aldehyde-fuchsin introduced by Gomori[1] into histological technique has been recommended by many investigators as a useful dye for staining elastic fibers, beta cell granules of the islets of Langerhans, certain basophilic cells (corresponding to beta cells of Romeis) in the anterior pituitary, and neurosecretory substances in the posterior pituitary of man and of various species of animals. Since aldehyde-fuchsin is not soluble in water, this dye is usually prepared as an alcoholic solution by admixing paraldehyde and hydrochloric acid into basic fuchsin dissolved in alcohol. In this method described by Gomori, it takes two or three days at room temperature until sufficient amount of deep purple aldehyde-fuchsin is formed in the mixed solution. This process of "ripening" has been explained by Bangle[2] that in the presence of acid catalyst, paraldehyde depolymerizes gradually to give acetaldehyde which reacts with the open amino groups of basic fuchsin. This reaction still goes on progressively after the dye mixture reaches the suitable state for staining, then leads to deterioration, and finally complete loss of selective stainability of the dye. Accordingly, when the staining with aldehyde-fuchsin is desired, the dye mixture should be made up two or three days in advance of each experiment, and repeated examinations of the staining properties are required during the period of ripening.

Attempting to avoid these disadvantages, Rosa[3] prepared aldehyde-fuchsin in dry form which was precipitated with chloroform from the three days old alcoholic dye mixture of Gomori. In the course of our study on the hypophysis of mouse, endeavors have been made to prepare aldehyde-fuchsin of identical stainability within more a reduced time, and we succeeded by starting the preparation with aqueous solution of basic fuchsin.

Experimental

Material

As the tissue material to test the staining properties of trial dye preparations, varieties of organs such as pancreas, hypophysis, trachea, lung, spleen, aorta and so forth of man, dog, cat and mouse were used. They were fixed with formalin, formalin-saline, sublimate-formalin, Zenker's fluid or Halimi's modification of Bouin's fluid[4], embedded in paraffin, and cut at 4–8 μ.

Preparation of aldehyde-fuchsin

At the beginning of this study, it was found that when the concentration of alcohol in Gomori's misture is reduced below 30%, precipitation of aldehyde-fuchsin takes place. Taking advantage of this insolubility of aldehyde-fuchsin in water, the authors have undertaken to introduce the preparatory principle of resorcin-fuchsin of Weigert into the preparation of aldehyde-fuchsin. Resorcin and ferric chloride were replaced with concentrated mineral acid and paraldehyde of various amounts, and the following formula was regarded as most satisfactory.

0.5% aqueous solution of basic fuchsin	100 m*l.*
Paraldehyde	3 m*l.*
Hydrochloric acid (sp. gr. 1.18)	1 m*l.*

The mixture was then kept in an oven adjusted at 55°C. After two or three hours, a dark violet precipitate was collected on a small filter paper, and dissolved in 100 m*l.* of 70% alcohol. Very satisfactory results were also obtained, when hydrochloric acid was substituted by the same volume of nitric acid (sp. gr. 1.42).

The precipitate is indistinguishable from that of resorcin-fuchsin in its appearance, but it is readily soluble in alcohol wihout heating. The solution is ready for use in staining procedure, and retains its specific staining properties for about two weeks.

In some preparations, in order to assure complete prevention of " overripening " of the dye, the excess aldehyde and acid were washed out from the precipitate prior to dissolving in alcohol. But this procedure was eventually omitted, since it resulted in considerable reduction of the specificity of the staining properties. Addition of hydrochloric acid into the 70% alcohol to dissolve the dye precipitate, as described by Rosa[3], should be also avoided, as it tends to produce a diffuse unspecific shade of the specimen.

Staining properties

With this preparation of aldehyde-fuchsin, a very intense and selective

staining of elastic fibers including the finest fibrils, beta cell granules in the pancreatic islets, certain basophils in the adenohypophysis, and neurosecretory substances in the neurohypophysis was obtained, after very slight oxidation of the sections with acidified solution of potassium permanganate. Some of these results are illustrated in Figs. 1–5. Certain mucus, mast cell granules and cartilage matrix are also very distinctly stained, but their affinity to the dye is not dependent on oxidation. As it will be mentioned below, pre-treatment of sections with Lugol's solution employed by Gomori is not successful. The most successful oxidant is a mixture of equal parts of 0.05% potassium permanganate and 0.5% sulfuric acid. A mixture of two parts of 5% potassium chlorate and one part of 8 *N* hydrochloric acid is also satisfactory, but the use of acidified solution of potassium bromate was unsuccessful.

Regarding the effect and significance of the oxidative procedure prior to staining, detailed studies have been made by Scott and Clayton[5] and Okamoto[6]. All of them confirmed in their results that elastic fibers, certain mucus, cartilage matrix and beta cell granules of the islets are stained without previous oxidation, and their staining intensity is conspicuously enhanced by pre-treatment of the sections with Lugol's solution. To our perplexity, our preparation of aldehyde-fuchsin did not stain elastic fibers and beta cell granules of non-oxidized sections, and even those in Lugol-treated ones, except for faint reaction of the internal elastic lamellae of arteries. On the other hand, the bluish-violet filtrate that remained after separation of aldehyde-fuchsin precipitate stained elastic fibers without any oxidation, though this tint was removed very rapidly after a short rinse in alcohol. But it was found finally, that addition of a small amount of paraldehyde into alcoholic solution of aldehyde-fuchsin precipitate brings out clear and distinct stainability of elastic tissues and beta cell granules in either non-oxidized or Lugol-treated specimens. However, this secondary addition of paraldehyde has been avoided, because it results in a rapid decrease of the selective stainability for pituitary basophils and pancreatic beta cells within a few days thereafter. Thus, the most simple and satisfactory procedure for the staining with this preparation of aldehyde-fuchsin may be summerized as follows:

1) The sections were deparaffinized and brought to water.

2) Dip in a mixture of equal parts of 0.05% potassium permanganate and 0.5% sulfuric acid for 2–4 minutes. Stop this oxidation while the color of the sections is still pale rose.

3) After a short rinse in distilled water, decolarize in 1% sodium metabisulfite.

4) Rinse under tap for several minutes.

5) Dip in 70% alcohol for 1 minute.

6) Stain in alcoholic solution of aldehyde-fuchsin precipitate for 5–30 minutes (for elastic fibers, 5 minutes; for beta cells of the islets and for neurosecretory substances, 10–20 minutes; for basophilic cells (beta cells of Romeis) of the adenohypophysis, 30 minutes).

7) Wash in 2–3 changes of 95% alcohol. If the background of tissue is moderately colored, differentiate with 70% alcohol containing one volume per cent of hydrochloric acid, and then transfer into 95% alcohol.

8) Dehydrate in absolute alcohol, clear in xylene, and mount in balsam.

After this procedure, in addition to the tissue elements listed above, many others as described by Scott and Clayton[5], Okamoto[6] and Halmi and Davies[7] are also stained.

Discussion

As various tissue elements represent more or less intense affinity to aldehyde-fuchsin, it is difficult to interpret the histochemical basis of this staining reaction. Concerning this problem, Soctt and Clayton[5] concluded that sulfuric acid groups and aldehyde groups are most responsible for the staining whether such groups may be derived by preceeding oxidation or not. As for pancreatic beta cells, they considered that previous oxidation splits disulfide bonds of cystine to give rise to sulfonic acid groups which may possess strong affinity to the dye. Regarding aldehyde groups, Bangle[2] stated that certain amino groups of aldehyde-fuchsin which remained free from the blocking with acetaldehyde may combine with aldehyde groups in the selective tissue elements. Recently, moreover, possible reactivity of aldehyde-fuchsin with carboxyl and phosphate groups is suggested by Graumann[8]. Halmi and Davies[7], on the other hand, described that their comparative observations on aldehyde-fuchsin staining, metachromatic activity and periodic acid-Schiff reactivity do not permit a conclusion about histochemical basis of aldehyde-fuchsin staining.

According to our observations, a certain part of aldehyde-fuchsin seems to be not insoluble in water. This is evident because the filtrate that remained after separation of precipitated aldehyde-fuchsin shows deep bluish-violet color. It is assumed that in this filtrate the following components of aqueous hydrochloric acid-paraldehyde-basic fuchsin mixture are contained: 1) A vast majority of acid and paraldehyde (a greater part of it may be in depolymerized state), and 2) a blue-violet dye which can react with elastic tissues, though it is very unstable. On the other hand, unless the specimens are previously treated with acidified potassium permanganate or chlorate, the water insoluble fraction of aldehyde-fuchsin

can stain nothing of the tissue elements, except those which contain large amounts of highly sulfated polysaccharide, e.g., cartilage matrix, mast cell granules or certain mucus. But under the co-existence of a small amount of paraldehyde, this fraction acquires distinct reactivity with elastic fibers and pancreatic beta cells without prior oxidation. This fact may bring forward a interesting suggestion about the staining properties of aldehyde-fuchsin itself. It is also reasonable, moreover, that the degradation of selective stainability is conspicuously delayed by the precipitating method described here, since aldehyde-fuchsin is assumed to be removed from the process of reaction with acetaldehyde as an insoluble fraction as soon as it is formed.

Summary

Aldehyde-fuchsin has been obtained in a precipitated form by adding paraldehyde into acidified aqueous solution of basic fuchsin. Alcoholic solution of this aldehyde-fuchsin precipitate shows a stainability very similar to that of Gomori's aldehyde-fuchsin mixture. Specific stainability for pituitary basophils, pancreatic beta cells and neurosecretory substances is retained about two weeks after preparation. As the time for the preparation is also markedly reduced, it has been made possible to carry out the preparation of aldehyde-fuchsin and the staining procedure within several hours.

References

1) Gomori, Am. J. Clin. Path., 1950, **20**, 665.
2) Bangle, Jr., J. Histochem. & Cytochem., 1954, **2**, 291.
3) Rosa, Stain Technol., 1953, **28**, 299.
4) Halmi, *ibid.*, 1952, **27**, 61.
5) Scott & Clayton, J. Histochem. & Cytochem., 1953, **1**, 336.
6) Okamoto, Wakayama Med. Rep., 1956, **3**, 9.
7) Halmi & Davies J. Histochem. & Cytochem., 1953, **1**, 447.
8) Graumann, Z. f. Zellforsch., 1957, **46**, 430.

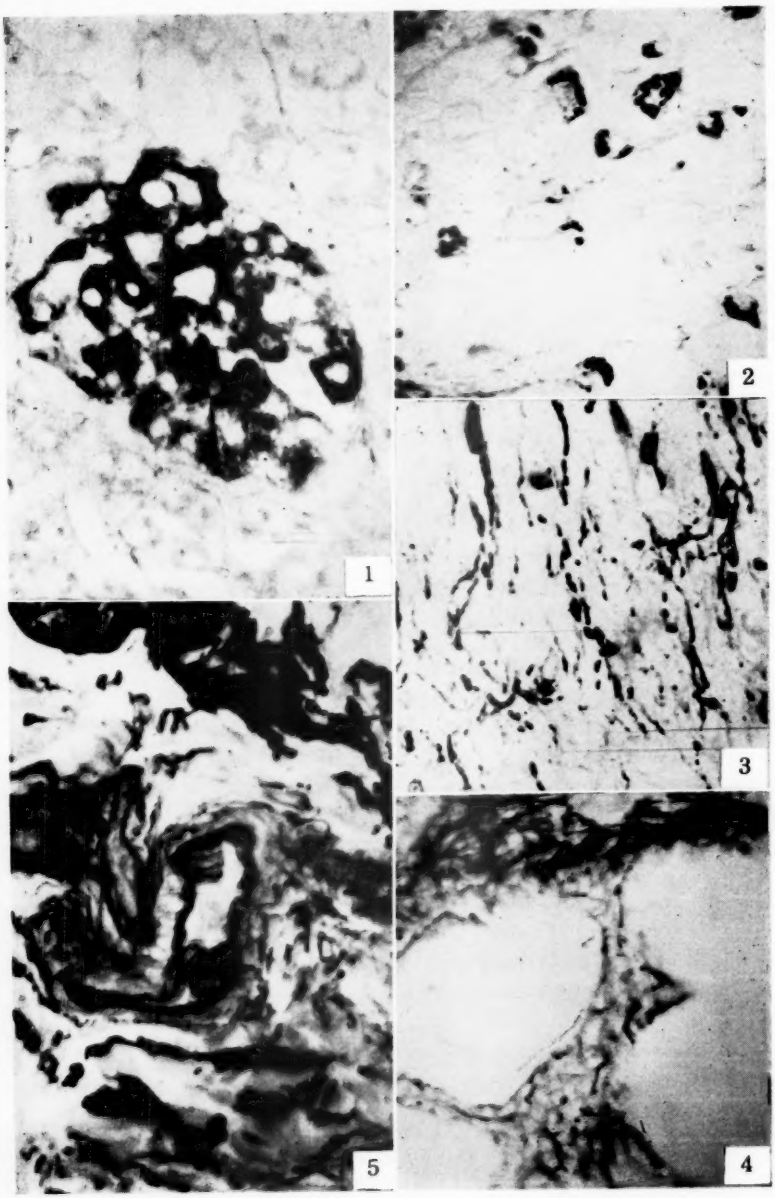

1
2
3
4
5

Fig. 1. Human pancreas, fixed with 10% formalin. Heavily stained beta cell granules of the islet stand out against a colorless cytoplasmic background. Nuclei remain unstained. Shade of the excretory cells is very faint.

Fig. 2. Central area of pars distalis of a male dog hypophysis, fixed with Bouin's fluid (Halmi's modification). Cytoplasmic granules of beta cells are selectively stained.

Fig. 3. Infundibular stalk of the same specimen as Fig. 2. Specific staining of the neurosecretory substances descending and retarded along the nerve fibers is shown.

Fig. 4. Lung of a new-born mouse, fixed with Zenker's fluid. Minute elastic fibers are scattered in interalveolar septa and around bronchioles. Other tissue elements are also slightly colored.

Fig. 5. Adventitial layer of the descending aorta of a male dog. Fixed with sublimate-formalin, and stained with nitric acid-paraldehyde-fuchsin. Fine elastic fibers in the walls of the arterioles (vasa vasolum) are also distinctly demonstrated.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Action Potential of the Atrio-ventricular Node (Tawara)*

Kojiro Matsuda†, **Takeshi Hoshi**† and **Shigenori Kameyama**
(松田 幸次郎) (星 猛) (亀山 重徳)

The Department of Applied Physiology, Tohoku University Medical School, Sendai

(Received for publication, April 25, 1958)

The membrane potential of the cells of the atrioventricular (A-V) node was investigated in the puppy by means of the ultramicroelectrode technique. Excised atrial preparations containing the A-V nodal region were used, under abundant supply of oxygen.

The characteristic features of the cellular action potential as follows were observed only in the localized area where the A-V node was known to exist. The characteristics are: 1) slow rise of depolarization, 2) definite delay of the spike from the start of depolarization amounting from 40 to 80 mSec; preceeding the spike there is a slow rise simulating the synaptic potential, at times a step-like potential, 3) the overshoot is small or absent, 4) slight undershooting following the repolarization and slightly lowered resting potential (Fig. 1). While the start of depolarization is almost simultaneous with that of the adjacent atrial fiber, the spike is delayed distinctly.

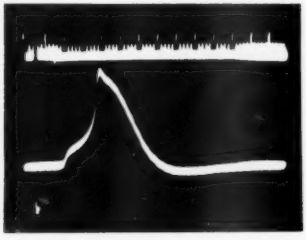


Fig. 1. Membrane action potential of the A-V node. Dog. Time mark: 10+50 mSec on the zero potential line. Voltage calibration: −100 mV.

Though it was not possible to trace the action potential changes from the A-V node to the bundle of His, the authors would consider that the "A-V conduction delay" is due to the spike delay of the action potential occurring in the course of the impulse conduction in a short distance at the proximal portion of the A-V node, owing to the characteristic behavior of the cellular membrane there.

* Reported in the 34th general meeting of the Physiological Society of Japan on May 26, 1957 at Kobe. † Present address: Department of Physiology, Faculty of Medicine, University of Tokyo.

Addenda. According to a recent, persona! communication from Dr. B. F. Hoffman, State University of New York, he noticed in the A-V node of the rabbit the action potentials which were low in amplitude and provided with a slow, notched rising phase. He supposed the decremental conduction at very low velocities.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

The Effects of ACTH and "Prae-hormon" on Electric Flicker

By

Yoshio Toratani
(虎渓 良男)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, February 17, 1958)

Motokawa and Iwama[1] found a great effect of oxygen lack upon the flicker sensation caused by electrical stimulation of the eye, and it was further found by Motokawa and Suzuki[2),3)] that the electric flicker could be used as a very convenient indicator for the measurement of fatigue, and then many investigations were carried out by this Motokawa's method of electric flicker. Takeyama[4)] reported that the value of electric flicker measured by this method increased in exposure to noise. Quite recently, Suzuki *et al.*[5),6)] reported that this method is very sensitive to noise and to fatigue caused by a steptest.

On the other hand, similar problems have been investigated by Thorn's test[7)] with special reference to the adrenocortical system. The number of eosinophil cells in the blood and 17 Ketosteroid (17 K.S.) in the urine are taken as indicators of the endogenic pituitary-adrenal activity. Recently, Nakamura[8)] investigated changes in eosinophil number following a steptest and Saito[9)] reported some significant chsnges in urinary 17 K.S. and blood eosinophil number when experimental animals, rabbits, were exposed to noise. Sakamoto[10)-12)] found a distinct diminution in blood eosinophil count and a decrease of urinary 17 K.S. in reponse to noise and during muscular labor, and showed that these changes could be prevented by intramuscular injections of ACTH.

In the present experiment the electric flicker value (EFV) was used in addition to urinary 17 K.S. and eosinophil number as indicators of the stresses caused by noise and by steptests, and the effect of ACTH and other hormones were investigated.

Experimental

Method

Ten healthy 20–40 aged subjects (5 males and 5 females) were used. Men-

strual phase was avoided in femal subjects, because EFVs generally increase in this phase[13].

An audiometer (51 A) was used as a generator of noise, and the noise level was kept always at 110 db. The distance between the origin of noise and the subject was 40 cm. Besides noise a pure tone of 500 cps and 70 db was used as a stressor. The tone was heard monaurally. Another stressor used was a step-test ; it consisted of going up and down at a constant rate two stairs, 25 cm. high each.

The subjects were injected ACTH (20 mg.) intramuscularly at 9.00 a.m. and Prae-hormon (500 R.U.) subcutaneously at 8.00 a.m.

Urinary 17 K.S. was measured 2 times, in the morning from 8.00 a.m. to 12.00 a.m. and in the afternoon from 12.00 a.m. to 6.00 p.m. with the method of Drekter and his associates[14].

The number of eosinophil cells in the blood was counted by Dunger's method[15] immediately before and after a stress.

The apparatus for electric flicker used in the present experiment was the one manufactured by Tôadempa & Co. after Motokawa's suggestion[16]. The stimulating current of the eye was increased from zero at a constant rate, 12 μA/sec., to determine the threshold for appearance of flicker (S_1), and the threshold for disappearance of flicker (S_2) was determined by decreasing the current from a sufficiently high level at the same rate as above. The difference between S_1 and S_2 obtained immediately before the stress, was denoted by ΔS_0, and that obtained immediately after the stress by ΔS. Each ΔS_0 and ΔS represent the mean of ten measurements.

Results

For 30 minutes before each experiment, the subjects were kept seated quietly. The three male subjects were exposed to noise of 110 db from 1.00 p.m. to 4.00 p.m. EFV, urinary 17 K.S. and blood eosinophil number were measured immediately before and after exposure to noise. The so-called urinary 17 K.S. before noise means the amount in the urine collected in the time interval from 8.00 a.m. to 12.00 a.m. and the so-called 17 K.S. after noise refers to the time interval from 12.00 a.m. to 6.00 p.m.

The results are summarized in Fig. 1. In this figure, white rectangles show the values obtained immediately before stress and black rectangles the values obtained immediately thereafter. As can be seen in B_1, B_2 and B_3 of this figure, the differences between white and black rectangles are remarkable in each subject (I, II and III). EFV immediately after stress increased by noise to twice the value obtained before stress. Urinary 17 K.S. and blood eosinophil number were decreased by noise significantly. Similar experiments were carried out 4 hours after an ACTH injection. The results are shown in C_1, C_2 and C_3 of Fig. 1. In this case, no significant difference could be found between the data obtained immediately before stress and those obtained after stress. Judging from any indicator, the effect of ACTH is obvious.

In the following experiment a steptest was used as a stressor in stead of noise.

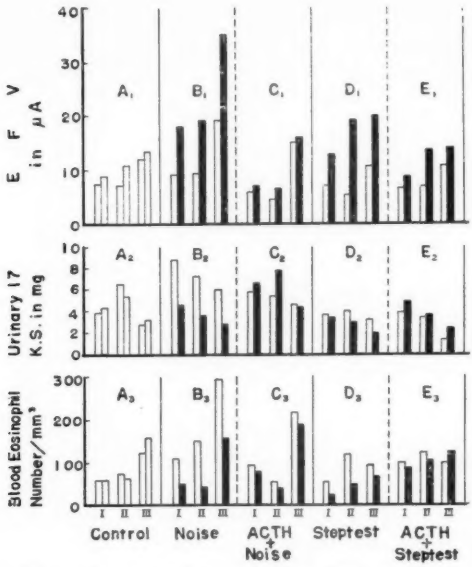


Fig. 1. Effects of noise and steptest upon EFV, 17 K.S. in urine and eosinophil cell numbers in blood, and preventive effects of ACTH. EFV in μA (upper), urinary 17 K. S. in mg. (middle) and blood eosinophil numbers per mm³ (bottom). White rectangles refer to values obtained immediately before stress and black ones to those obtained immediately after stress. Results from 3 subjects are denoted by I, II and III.

The rate in the steptest was such that it required 4 seconds to go up and down the step each time. The steptest was continued for 40 minutes. The results obtained are represented in Fig. 1 D_1, D_2 and D_3. As can be seen in this figure, EFV increased from 6.9 to 12.6 μA in subject I, from 5.7 to 18.9 μA in subject II and from 11.0 to 20.3 μA in subject III, and these changes are comparable with those caused by noise (cf. B_1, B_2 and B_3 in Fig. 1). As can be seen in D_3, the eosinophil number immediately after stress is smaller than a half the value immediately before stress in 2 subjects. But the change in urinary 17 K.S. was not to remarkable as with the other indicators (see D_2). These effects of the steptest were greatly reduced by ACTH injection, as can be seen in E_1, E_2 and E_3. It is to be noted that 17 K.S. increased slightly after the test (see E_2).

As control similar experiments were performed without any stress. A_1, A_2 and A_3 in Fig. 1 represent EFV, urinary 17 K.S. and blood eosinophil number for three subjects in the control experiment. The difference between two white rectangles for each subject indicates the degree of experimental error.

Next, the effect of Prae-hormon was investigated. At 8.00 a.m. ten healthy subjects (5 males and 5 females) were injected Prae-hormon (500 R.U.) subcutaneously, and each subject so treated was exposed to noise of 110 db for 25

TABLE I

Changes of EFV in μA in Exposure to Noise (11° db) for 35 Minutes after Prae-hormon (500 R.U.) Injection

Subject-number and sex	Control			Values 4 hours after Prae-hormon injection					
				1st day			2nd day		
	Before noise (ΔS_0)	Immediately after noise (ΔS)	Difference ($\Delta S-\Delta S_0$)	Before noise (ΔS_0)	Immed. after noise (ΔS)	Diff. ($\Delta S-\Delta S_0$)	Before noise (ΔS_0)	Immed. after noise (ΔS)	Diff. ($\Delta S-\Delta S_0$)
1 (♂)	3.6	14.4	10.8*	4.8	7.7	2.9	4.7	7.1	2.4
2 (♂)	10.7	18.0	7.3*	6.8	8.7	1.9	6.3	7.5	1.2
3 (♂)	15.0	24.9	9.9*	14.3	17.6	3.3	14.3	17.3	3.0
4 (♂)	26.7	38.7	12.0*	24.6	27.9	3.3	27.0	29.3	2.3
5 (♀)	9.8	16.8	7.0*	9.8	10.7	0.9	12.3	14.0	1.7
6 (♀)	14.9	27.0	12.1*	18.6	24.8	6.2*	16.4	15.3	−1.1
7 (♀)	2.1	8.7	6.6*	4.4	6.2	1.8	5.1	6.2	1.1
8 (♀)	31.2	45.2	14.0*	31.4	37.7	6.3*	40.7	43.2	2.5
9 (♀)	42.0	57.8	15.8*	57.6	56.7	−0.9	55.1	58.8	3.7
10 (♂)	3.6	9.9	6.3*	2.3	2.7	0.4	3.3	4.0	0.7
Average			10.38			2.61			1.75

* · · · Statistically significant.

minutes from 12.00 a.m. The date obtained immediately before and after exposure to noise are summarized in Table I. In control experiments in which no administration of Prae-hormon was made, there were great differences between the values obtained immediately before (ΔS_0) and after (ΔS) exposure to noise. It is without saying that these differences are statistically significant. On the first day of Prae-hormon injection, the effect of noise, the difference $\Delta S-\Delta S_0$ was found decidedly lower than that in control. Namely, the average of $\Delta S-\Delta S_0$ in control was 10.38 μA, while the average of $\Delta S-\Delta S_0$ for the first day of Prae-hormon injection was only 2.61 μA. Similar experiments were carried out on the following day. On the second day of treatment with Prae-hormon, the average of $\Delta S-\Delta S_0$ was only 1.75 μA. That is, the effect of Prae-hormon was more marked on the second day. From these findings, it may be said that the influence of noise is prevented by Prae-hormon injection.

From the latter experiment it is likely that Prae-hormon has a cumulative effect. This was confirmed by the following experiments. As stressors a pure tone of 500 cps and 70 db and a steptest were used. The subject was injected Prae-hormon (500 R.U.) subcutaneously at 8.00 a.m. on five successive days. Every day from 12.00 a.m. the subject was exposed to the continuous tone for 10 minutes, and during this period and thereafter EFVs were measured repeatedly. The result obtained is shown in Fig. 2A, in which the numbers 1, 2, 3, 4, and 5 refer to the first, the second, the third, the fourth and the fifth

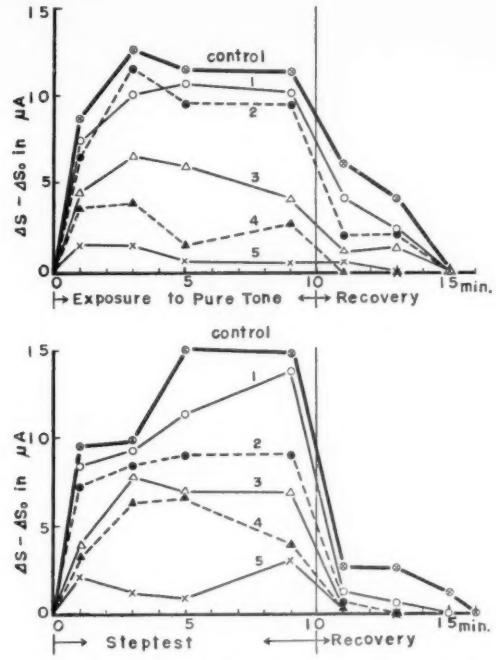


Fig. 2. Change of EFV (μA) in exposure to pure tone of 500 cps and 70 db (A), and that in steptest (B). Abscissae: Time of stress and recovery. Number by each curve indicates number of days of treatments with Prae-hormon (one injection a day).

day respectively. Fig. 2B represents the results obtained with steptests as stressor. The steptest continued for 10 minutes, the rate of going up and down the step being 20 per minute. As can be seen from Fig. 2A and B, EFVs became progressively lower day by day, and it seemed that the stressors used would have almost no effect after 5 days' treatment with Prae-hormon.

Discussion

As stated in Selye's theory, noise can act as a stressor and cause the so-called alarm reaction, in which decreases in urinary 17 K.S. and in the eosinophil cell count occur. This is evidenced by the present investigation; as can be seen in Fig. 1, remarkable decreases in 17 K.S. and in the eosinophil number were caused by noise of 110 db enduring for 3 hours. It deserves special attention that EFVs showed a significant response to the stress. The response of EFVs was always so clear-cut that a positive

response was seen with regard to EFVs even in cases the other indicators failed to show a positive response. The preventive effects of ACTH and Prae-hormon could be shown cleary with EFVs as well as by the routine methods with 17 K.S. and blood eosinophil numbers. The effect of ACTH was found to be prompt, but it took certain time to obtain a full effect of Prae-hormon (see Fig. 2). The reason is apparent from the properties of these hormones. The decrease of adrenal cortex hormone caused by a stress is said to be a secondary effect following a decrease in secretion of ACTH (Sakamoto[12]). As has been observed above, a slight increase in urinary 17 K.S. was caused after an ACTH administration (see Fig. 1 C_1 and E_2). This would be due to overcompensation of the hormone in question.

It is known that an increase of EFV occurs under various unfavorable conditions such as insufficient oxygen supply, menstruation, Roentogen ray irradiation, bleeding, fatigue after physical and mental efforts, etc. In this way changes in EFVs are not specific to any causative factor. This non-specific character of the increase in EFVs may most easily be interpreted when it is regarded as one of the alarm reactions and related to the hypothalamus-pituitary-adrenocortical system[17),18)].

In the present investigation it was shown that the EFV is most sensitive to various kinds of stress, and that the change in EFVs is prevented by ACTH and Prae-hormon. These facts provide evidence for the above-mentioned interpretation. The method of EFV is so practical that it would be of great value, if this method is utilized for study of the alarm reaction and related problems.

Summary

By the method of electric flicker, the effects of various stressors, such as noise, exercise, etc. were investigated with special reference to the pituitary-adrenocortical system. The subjects used were healthy 5 males and 5 females.

1. Electric flicker values (EFVs) were measured immediately before and after exposure to noise of 110 db lasting for 3 hours. For comparison, urinary 17 K.S. and blood eosinophil numbers were measured in parallel. EFVs increased significantly by noise, while significant decreases were found in urinary 17 K.S. and eosinophil count.

2. These changes could be prevented by ACTH (20 mg.) administered intramuscularly.

3. As a stressor, a steptest was used in stead of noise, and similar results were obtained.

4. It was shown that Prae-hormon (500 R.U.) had a preventive

effect, too, but its cumulative action was remarkable; the increase in EFVs in response to a strong pure ton (500 cps and 70 db) was as remarkable as in control on the first and second days of Prae-hormon administration, but the response became progressively smaller as the same treatment with Prae-hormon was repeated further.

5. This relation was confirmed further with a steptest as a stressor. From these facts, it was concluded that the EFV is a sensitive indicator of the general reaction to stress in the sense of Selye.

The author has pleasure in acknowledging his indebteness to Prof. K. Motokawa who has guided him kindly.

References

1) Motokawa, K. & Iwama, K., Tohoku J. Exp. Med., 1949, **50**, 319.
2) Motokawa, K. & Suzuki, K., Jap. Med. J., 1942, **1**, 200.
3) Motokawa, K. & Suzuki, K., Nisshin Igaku (Jap.), 1948, **25**, 523.
4) Takeyama, K., J. Oto-Rhino-Laryngolog. Society (Jap.), 1956, **9**, 47.
5) Suzuki, K., Yamashita, H., Megumi, Y. & Kato, H., J. Science of Labour (Jap.), 1956, **32**, 247.
6) Suzuki, K., Toratani, Y., Inoue, T. & Oiso, T., *ibid.*, 1957, **33**, 494.
7) Thorn, G. W., J.A.M.A., 1948, **137**, 1005.
8) Nakamura, S., Nisshin Igaku (Jap.), 1955, **42**, 412.
9) Saito, S., J. Physical Society of Japan, 1957, **19**, 1069.
10) Sakamoto, H., J. Science of Labour (Jap.), 1956, **32**, 1005.
11) Sakamoto, H., *ibid.*, 1957, **33**, 93.
12) Sakamoto, H., *ibid.*, 1957, **33**, 175.
13) Umetsu, T. & Suzuki, K., Tohoku J. Exp. Med., 1957, **65**, 237.
14) Drekter, I. J., Heisler, A., Seism, G. R., Stern, S., Pearson, S. & McGavack, T. H., J. Clin. Endocrinol., 1952, **12**, 55.
15) Bonner, C. D., J.A.M.A., 1952, **148**, 634.
16) Motokawa, K., Umetsu, J., Kobayashi, M. & Kameyama, M., Tohoku J. Exp. Med., 1956, **64**, 151.
17) Selye, H., Canad. Med. Assoc. J., 1936, **34**, 706.
18) Selye, H., Annual Report on Stress. Montreal, Acta. Inc., 1950.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Action of Acetylcholine and Adrenaline upon the Membrane Potential of the Atrio-ventricular Node (Tawara)*

Kojiro Matsuda†, Takeshi Hoshi† and **Shigenori Kameyama**
(松田 幸次郎) (星 猛) (亀山 重徳)

The Department of Applied Physiology, Tohoku University Medical School, Sendai

(Received for publication, April 25, 1958)

For the purpose to elucidate the mechanism of the dromotropic actions of the vagus and sympathetic nerves upon the A-V conduction, the effects of acetylcholine and adrenaline on the cellular action potentials of the A-V node[1] of the puppy were investigated.

As shown in the Fig. 1, acetylcholine depressed the rate of rise of action potential, prolonged the delay of the spike until the spike became extinguished leaving only a minute local depolarization (A-V block). Such effect of acetylcholine is not like to that upon the ordinary atrial fiber. The effect of adrenaline was opposite to the effect of acetylcholine: increase of action potential rising velocity, decrease of the spike delay, slight increase of spike height.

Both of these effects were quite reversible as well as reproducible.

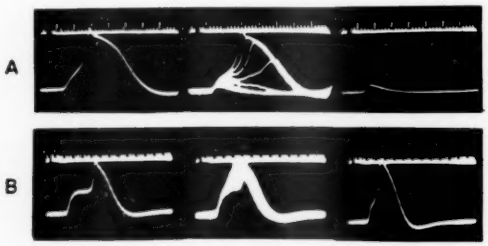


Fig. 1. Effects of acetylcholine (A) and adrenaline (B) on the membrane action potential of the A-V node. Left: control, middle: successive changes superimposed, right: final stage. Dog.

References: 1) Matsuda, Hoshi and Kameyama, This journal, 1958, **68**, 8.

* Reported in the 34th meeting of the Physiological Society of Japan on May 26, 1957 at Kobe. † Present address: Department of Physiology, Faculty of Medicine University of Tokyo.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Histochemical Study on Placental Permeability

By

Ryuji Yamaguchi, Kiichiro Noda, Gisaburo Sugawara
(山口 龍二) (野田 起一郎) (菅原 義三郎)
Haruo Murakami, Yujiro Yamada, Toru Yamashita
(村上 治夫) (山田 雄二郎) (山下 徹)
Shuji Akiyama, Yoshihiro Okuda and
(秋山 修爾) (奥田 宜弘)
Hiroshi Imamura
(今村 寛)

From the Department of Obstetrics and Gynecology, Faculty of Medicine, Tohoku University, Sendai;
Director: Prof. K. Kushima

(Received for publication, February 25, 1958)

For interpreting the mechanism of matter exchange in the placenta, the old-standing controversy whether it is effected by physical diffusion alone or some biological activity comes in is as yet unsettled. The camp advocating the former theory take the observations that the gradient of concentration of small-molecular substances between the mother and the fetus is in most cases ascending toward the maternal side and that in administration tests the permeation of the administered substances shows a velocity approximating the theoretical value according to the diffusion law as the basis of their opinion (Bickenbach and his school[1]), while the latter opinion is supported by the researchers who point out that some of the materials are found in higher concentration in the fetal side, that upon administering different but constitutionally similar substances a difference in permeability and that some selectivity or positive activity must be postulated beside mere diffusion in interpreting the mechanism of transition of antibodies and some histochemical findings (Cunningham[2], Effkemann[3], Chinard[4], Widdas[5], Huggett[6], Page[7]). Few are the contentions, however, based on confirmed observations on the actual migration of substances. In consideration of such a state of affairs, the present authors undertook the experiments of administering various substances to the placenta for following up the matter exchange between the maternal and the fetal tissues availing ourselves of the new histochemical methods. The findings obtained are reported in the following.

Experimental

Methods and Materials

The placentae used in this study were sampled from women in various stages of normal pregnancy (upon term delivery and artificial abortion) and rabbits, rats and mice in the last stage of pregnancy.

The administration of the substances tested was chiefly effected by one of the following processes:

1) Intravenous injection in the maternal body just before delivery or in animals, just before laparotomy or sacrificing; 2) intra-aortal injection in animals; 3) repeated intravenous injection in animals; 4) incubation of human placentae of various stages of pregnancy, in isotonic solution, when possible, of the administered substances for five minutes to several hours at 37°C, and 5) perfusion of expelled placentae upon mature delivery with solutions of substances through the umbilical artery.

The substances used in this study, their form for application and the staining methods were as follows:

1) *Copper*[8]. $CuSO_4$ was used in 0.05% solution (most frequently in physiological saline) for infusion of human placentae. 200 cc. of such a solution was used in injecting maternal bodies. 0.5% solution of the commpound was used in treating animals. For staining out copper, Okamoto-Utamura's rhodanine method and the rubeanic acid method were applied.

2) *Iron.* 0.03% solution of ferric gluconate in physiological saline. Staining with Prussian blue or by Turnbull blue method.

3) *Calcium*[9]. Solution of $CaCl_2$. 40 cc. of 2% solution for injection in maternal veins, and 5–10 cc/kg. of the same for rabbits and mice. For incubation 1.5% $CaCl_2$ solution was mixed with physiological saline in different ratios to prepare isotonic solutions containing calcium in the range of 5–400 mg/d*l*. For perfusion through the umbilical artery, a solution in physiological saline containing 100 mg/d*l*. of calcium was used. Staining was done following a newly devised variation of Kóssa's method*, Okamoto-Senoo-Kato's alizarine-S method and purpurine staining.

* The methods hitherto in use being serviceable only in demonstrating Ca deposits, we devised the following variation. The tissue samples containing water-soluble Ca were immersed in 10% of formalin solution containing 4–5% of Na_2HPO_4 to change the water-soluble calcium compound int › unsoluble $CaHPO_4$ and at the same time to fix the specimens and then the Kóssa's method was applied to detect Ca.

4) *Fat.* The fat injection solution Fatgen** was used, in original strength for rats and in 5% solution in physiological saline for human placentae. For staining out, the Sudan III (Daddi's method) was chiefly applied, the Goldmann's, the Sudan black, the Nile blue and Smith-Dietrich's lipoid staining being also used in combination.

** Fatgen is an emulsion containing 20% of sesame oil and 7% of glucose, consisting of triglycerides of linol, olein, stearin, parmitin, myristin and other fatty acids. The diameter of the fat granules in it is always below 0.5 μ (Higasa, *et al.*[12]).

5) *Steroid hormones*[10]. Mice were injected intravenously with 5 mg., 5 mg. and 3 mg. respectively of progesterone, testosterone or estrone dissolved in 0.2–0.3 cc. of mixture of equal parts of 40% ethylurea and 40% ethylurethan solutions per head. For staining, Ashbel-Seligman's method was used and Sudan III for control staining.

6) *Glucose*[11]. To the maternal bodies, 100 cc. of 50% glucose solution was administered just before delivery, and for the incubation tests, 5% glucose solution was used. For staining, we adopted the Okamoto-Kadota-Aoyama's silver nitrate method and toluidine blue staining, and also the Best's carmine method for staining out glycogen.

Results

1) *Copper*[8]. In the experiments of perfusing of umbilical artery, this element was found arranged in net-work stretching from the villous capillaries into the stroma, in lines running beneath the syncytium cells and in granules or diffused patches within these cells. More rarely we saw the picture of copper stains passing out from the syncytium cells into the intervillous spaces. After incubation, the finding was rather widely variable by the positions of the villi or by placenta, but generally speaking, copper was found in diffused existence in the syncytium cells, in linear arrangement beneath the syntcyium cells and in reticular alignment in the stroma, the last net-works sometimes showing the appearance of being in contact with the blood capillaries (Fig. 1). When injected into the maternal vein just before parturition, copper was found somewhat increased in the decidua but entirely absent in the villous part. When pregnant rats were injected, it was detected in granules in the syncytium cells and in reticular arrangement around the fetal blood vessels, particularly well in the cases examined 5–30 minutes after injection.

In the cases examined 24 hours after repeated intravenous injection, almost no copper was detected in the syncytium cells, the villous stroma etc., but in a relatively large quantity in the epithelium of the yolk sac. In the rabbits intra-aortally injected, the same findings as in the intravenously injected animals came forth in a higher degree and besides, a strong diffuse reaction of copper was found in the fetal erythrocytes.

Copper, as well as all the other substances tested, was found in linear and reticular arrangements as given above where the villous epithelium was normal, but in the part where the epithelium was in fibrinoid degeneration, the administered substances were found largely accumulated in the fibrinoid loci but very rarely transported thence into the stroma.

2) *Iron.* It is known since sometime ago that iron is accumulated beneath the epithelium and reticularly arranged in the stroma without external administration (Hofbauer[13]), but after incubation in ferric solution, such findings were found extremely enhanced.

3) *Calcium*[3]. When human placentae were incubated as above, calcium was detected adhering moss-wise on the surface of the chorionic epithelium and in linear arrangement in the vicinity of the basal layer beneath the syncytium

cells, but scarcely in the syncytium cells themselves. In the placentae from the early stage pregnants, it was found in lines filling the intercellular spaces of the Langhans cells and thence stretching into the basal part of such cells. In the Langhans cells themselves, however, no calcium was detected. The quantity of calcium passing beyond the basement membrane into the stroma was considerably lower, but in such cases the element was distributed always in linear or reticular arrangement, seemingly following the courses of the argyrophilic fibrils (Figs. 2 and 3). Such a penetration of calcium into the stroma was effected in an extremely short time (within 5 minutes), but the quantity thus entering the stroma was little affected by lapse of longer time and a heightening of calcium concentration in the incubating solution resulted only in an increase of the calcium content of the syncytium cells but no perceptible rise in its transitioin into the stroma. Such findings were found independent of the age of the fetus and nearly identical findings appeared in placental villi killed with corrosive sublimate. The moss-like deposit of calcium on the villous epithelium, when once incubated and then immersed in pysiological saline, disappeared within a short time. In the tests of perfusing expelled placentae through the umbilical artery, calcium was seen infiltrating out of the peripheral villous blood vessels into the surrounding tissue and then running through the stroma in net-like courses and accumulating in lines beneath the villous epithelium, but penetrating only in very small quantity into the syncytium cells through the basement membrane. Scarcely any trace of calcium penetrated into the placenta was observed in the tests of intravenously administering calcium to the expectant mothers.

4) *Fat.* After intravenous injection in pregnant rats, fat begun to be demonstrated in the villous blood vessels in 15 minutes and in 30 minutes the fat content reached the maximum level, being found in the trunk blood vessels in the villi too. The fat granules transferred into the fetus were found considerably smaller in size than those remaining in the maternal blood vessels. In human placentae of earlier stage incubated in fatty emulsion, fat appeared as if somewhat increased in the syncytium cells, the trophoblast buds, the cell islets and in the stroma, but no infiltration of fat was ever observed in placentae from later stage gravidae. After perfusion through the umbilical artery, fat filled up the vascular lumina but not a particle was found passing over into the stroma or the villous epithelium. When 25% in volume of bovine bile was mixed with fatty emulsion before incubation, fat begun accumulating on the surface of the syntytium cells and in one hour passed over into the syncytium cells and the stroma. In this experiment, the fat granules were found arranged in lines in the basal part of the syncytium cells, but the quantity of fat in the stroma was lower and the reticular arrangement was not definite. In the perfusion tests through the umbilical artery too, addition of bile facilitated the passage of fat through the capillaries into the stroma, so that the fat granules came into line formation beneath the syncytium cells and partly penetrated further into the cells (Figs. 4 and 5). In all the above cases, staining for fatty acids always gave negative results, so we might assume that fat is not decomposed into fatty acids in these cases but only pass through the placental barrier when atomized into

very small granules. Considering, however, the possibility of the added bile affecting the permeability of the chorionic epithelium or the fetal capillary walls, we added Indian ink to the bile before repeating similar tests, but this pigment never entered the specimen in incubation test and in a very small quantity in the umbilical artery perfusion tests, in a quantity similar to that found in the perfused placenta in the same test minus bile.

5) *Steroid hormones*[10]. When mice in later stage of pregnancy were intravenously injected with solution of steroid hormones, in 30 minutes a very perceptible increase of such hormones was ascertained in the entire labyrinth of the villi, and in the same position as in the controls, positive granules were found in linear arrangement beneath the syntcyium cells and increased in the stroma and the epithelial cells of the yolk sac. One hour after the injection, those granules began to decrease. Upon histological examination, such positive particle were found increased in a very small quantity in the fetal liver, subcutaneous tissue and muscles, so it may be inferred that steroids, as the other substances tested, penetrated in quantity as far as the villous epithelium, but only little of the hormones go further.

6) *Glucose*[11]. In placentae sampled 30–40 minutes after injection in maternal veins, positive glucose granules were frankly increased in the syncytium cells and in a somewhat larger quantity than in the control specimens from untreated subjects in the stroma, but after one hour the difference between the tested and the control specimens was nullified. In the placentae from rabbits intravenously injected with 20 cc. of 50% glucose solution, a few positive granules were found in the syncytium cells but none in the stroma. In the placental specimens sampled from human gravidae in the last stage of pregnancy and incubated in glucose solution, we observed a slight increase in the layers of syncytium cells, distinct linear arrangement of glucose granules in the vicinity of the basement membrane and reticular formations of them in the stroma.

Discussion

Summarizing and reviewing the above results, we may state as follows:

In incubation tests, the administered substances seem first to accumulate on the surface of the epithelium of villi and then to penetrate into the cell bodies. This epithelial surface, examined under an electron microscope, is found to be densely overgrown with microvilli, so that its superficial area is very large (Isomura[14], Wislocki and Dempsey[15], Sawazaki *et al.*[16]). The apparent moss-like accumulation of examined substances described above, therefore, may not be necessarily due to adsorption of these substances onto the surface of the epithelium, seeing that the complicated surface structure of this part may hinder the washing out of the substances from this part in the process of staining and thus may give the appearance of moss-like deposits. But when we recall that such surfacial accumulation was found

less prominent when the tested villi had been killed with sublimate, we may assume the existence, if not of a certain biological activity, at least some adsorptive action originating in the mere fact that this part is alive. Since, however, we have not experienced such picture outside our experiments with all substances tested and we could not conduct such experiments with the help of an electron microscope, we cannot be as yet sure of our inferences.

No mentionable finding could be obtained concerning the state of existence of the tested substances in the syncytium cytoplasm, so of course we cannot make inferences whether the substances are in physically or chemically changed form or not in the cell bodies. We can only say that the tested substances in the cytoplasm were very often in the form of very minute granules not to be found in the other positions and were only small in number. The histochemical methods used in this study of ours could serve in detecting uncompounded particles of the tested substances, and if such substances are combined into polymers, especially with protein molecules, in the syncytium cells, these compounds would have been overlooked in all probability, and also in the case when the concentration of the tested substances in the syncytium cells was near the limit of detection, the finding we have described above would have been the natural result. Besides, the existence of a carrier system alluded to by Chinard *et al.*[4], Widdas[5], and Page[7] could be only surmised at in our present study.

It is not that we have for the first time obtained the picture of the tested materials accumulated in a large quantity in the vicinity of the villous epithelium and the basement membrane, but formerly Hofbauer[13] reported on such accumulation of iron and Schönig[18] on that of calcium, but these authors interpreted their findings in the light of reserve deposits also or even mere artefacts formed in the staining process in them, scarcely paying attention to the problem of the permeation mechanism in this connection. Our experimental results, however, seem to suggest that these parts have a very high importance in the process of matter exchange between the maternal and the fetal tissues. Thus, whether the tested substances are administered by incubation or perfusion through the umbilical artery, these substances are always first accumulated in these parts and then are transferred into the stroma or the syncytium cells, but the quantity thus transferred is only a minute part of the accumulated substances in the epithelium or the basement membrane. To interpret this phenomenon, we may suppose either that 1) the permeating substance form a picture of adsorption in these parts during the process of fixing and staining the specimens, that is, product of artefact, or that b) these parts possess a selective function in the process of mother-fetus matter exchange. The former opinion is not be entirely refuted when we consider the defects unavailable

in applying the histochemical techniques presently available, but if these findings were always artefact, similar pictures should appear in all the other parts of the specimens too, and seeing that such accumulations were found with nearly all the substances we tested, we may fairly deny that these were mere artefacts. In connection with the interpretation b) above, the fact that in our incubation tests with calcium, and increase in the administered dosis was followed by a nearly proportionate increase of the element in the syncytium cytoplasm but the quantity thence passing over into the stroma was not at all increased in a comparable ratio, and the observation by Wislocki and Dempsey[15] of a stout basement membrane beneath the trophoblasts obtained an electron microscope lead us to suppose that these parts are in possession of some controlling or selective function against the changes in the external concentration. To the question what is the nature of this selectivity we cannot as yet make any definite answer based on what we have learnt to date, but we can point out at least that, as shown in our tests with fat, the larger original fat granules cannot pass the placental membrane but when the granules are atomized into smaller particles by addition of bile, they can easily pass beyond, and that as shown in the experiments with pigments by Goldmann[19] Wislocki[20] and Sawazaki *et al.*[16], the pigment particles in size of the order of those used in supervital staining can penetrate into the syncytium cells but do not pass further into the fetal side, and these findings seem to suggest that the grain size of the administered substances is one of the factors controlling the selective function. In this connection, it may be objected that, in spite of the very low molecular weights of iron, copper and calcium compounds, we found in our tests that these elements too were blocked at these part, but as ionized substances are usually found compounded with protein in living organisms, we may take that our tested metal elements formed compounds with protein in the cells and the grain size of such polymerized compounds were too large for permeation. In reference to the opinions of Wöhler[17] who presumed a participation of ferritin in the process of transportation of iron and, of Chinard *et al.*[4], Widdas[5] and Page[7] who postulated the existence of a carrier system for facilitating permeation of sugars and amino-acids, which acts in regulating the permeating quantity by their height of concentration, may we not point to these part as the field of the mucosal block?

Regarding the question " Is the selective action of these part of biological or physical nature? ", we might for the time being at least disregard the probability of biological function, for we have seen this selectivity active in the villi killed with corrosive sublimate as well, but we must wait for the outcomes of further experiments to decide on this point. The

results of the works by Hoskins and Snyder[21], Furuhashi[22], Sato[23] and Bogert and Plass[24] pointing to the conclusion that the selectivity of the placenta admits the transition of calcium fron the maternal to the fetal side but not at all or only very scarcely *vice versa* may be not precisely accurate in all particulars, but such phenomenon can also be explained by assuming the existence of selective function in the epithelial cells of the villi and in the vicinity of the basement membrane, and this assumption also facilitates the interpretation of the phenomenon that some substances are more highly concentrated on the fetal side.

In our study, we found that the Langhans cells are not at all interested in the permeation of our tested substances which only passed through the intercellular spaces. Accumulation of the substances, however, came forth in the basal part of the Langhans cell layer, and thence the substances made transition into the stroma, except in the placentae from the later stage gravida, where the transition went out from the accumulation below the syncytium layer. Our results gave the impression as if the Langhans cells have some other part to play outside the process of matter permeation —perhaps a part in the production of hormones.

The matter transportation in the placental stroma in our tests was almost always observed to be effected along the stromatic fibres. This fibre formation, as examined to represent the course of argyrophilic fibrils. It has been known since comparatively olden time that when iron, calcium and such substances are histochemically searched for, these are usually found in fibre formation, but we regret tht most of the past researches looked upon it only in the light of artefacts and rarely, if ever, saw in it a picture of intra-stromatic matter transportation. But since the transportation lines always start in the epithelium or the capillaries of the villi and connect these two formations, particularly markedly in samples of which the epithelium had been injured during sampling, whether the substances tested were administered through the maternal or the fetal side, and the formation was observed also in the tests using Indian ink independent of fixing and staining, we are inclined to presume that these fibrous lines represent an extra-vascular transport system of fluid as claimed by Kihara[25] and partially suggested also by Tatsumoto[20] and Sawazaki *et al.*[16]

The above statements are wholly based on the results obtained by histochemical methods, and of course we cannot say that we have herewith succeeded in explaining the entire mechanism of matter exchange between mother and fetus in the placenta in all its particulars. Future studies with electron microscope, physical and chemical methods are yet to be expended on the subject. We are, however, rather skeptic against the opinion of

Bickenbach and his school[1] who strongly contend that nearly all the matter exchange processes are cased on physical actions of diffusion and osmosis. We believe that the villous epithelium is not a mere semipermeable membrane but indubitably possesses a rather powerful selective function.

Conclusion

1. The present authors have histochemically followed up the mechanism of matter permeation in the placenta, especially its villi, using metallic salts, fat, steroid hormones and glucose as tracer.

2. The substances administered from the maternal side first accumulate on the surface of the chorionic epithelium and thence pass over into the syncytium cells and the villous stroma, but the basement membrane of the chorionic epithelium constitutes a barrier with a certain selectivity and controls the permeation of the administered substances. The same mechanism is in action when the substances have been administered from the fetal side.

3. The transportation of substances in the stroma of the villi is effected through the extra-vascular fluid transportation system consisting of argyrophilic fibrils.

References

1) Bickenbach, Seitz-Amreich's Biol. u. Pathol. d. Weibes., Vol. VII, Part I, Urban & Schwarzenberg, Berlin-Insbruck-München-Wien, 1952, pp. 219–238.
2) Cunningham, Am. J. Physiol. 1920, **53**, 439; *ibid.*, 1922, **60**, 448.
3) Effkemann, Z. Geburtsh., 1938, **117**, 405.
4) Chinard, Danesino, Hartmann, Huggett, Paul & Reynolds, J. Physiol., 1956, **132**, 289.
5) Widdas, *ibid.*, 1952, **118**, 23.
6) Huggett, Am. J. Obst. & Gyn., 1955, **69**, 1103.
7) Page, *ibid.*, 1957, **74**, 705.
8) Sugawara, Nippon Sanka-Fujinka Gakkai Zasshi (Jap.), 1958, **10**, in press.
9) Yamashita, *ibid.*, in press.
10) Akiyama, *ibid.*, in press.
11) Imamura, *ibid.*, in press.
12) Higasa, Asada, Zaitsu, Tsukada & Nakada, Nippon Geka Hokan (Jap.), 1952, **21**, 1.
13) Hofbauer, Hoppe-Seyler's Zeitschrift für Physiologische Chemie, 1903/1904, **40**, 240; Gründzüge einer Biologie der menschlichen Plazenta, Braumüller, Wien-Leipzig, 1905.
14) Isomura, San-Fujinka no Sekai (Jap.), 1950, **7**, 781.
15) Wislocki & Dempsey, Anat. Record, 1956, **123**, 133.
16) Sawazaki, Mori, Ota, Hiraoka, Nawa, Harada, Niimi & Inoue, Hormone to Rinsho (Jap.), 1956, **4**, 1081.
17) Wöhler, Dtsch. med. Wschr., 1955, **80**, 30.
18) Schönig, Z. Geburtsh. u. Gynäk., 1929, **94**, 451
19) Goldmann, Halban-Seitz's Biol. u. Pathol. d. Weibes., Vol. VI, Part I, Urban & Schwarzenberg, Wien-Berlin, 1925, p. 207 (stated by Dietrich); Beitr. klin. Chir., 1912, **78**, 1.
20) Wislocki, Anat. Record, 1921, **21**, 29.

21) Hoskins & Snyder, Am. J. Physiol., 1933, **104**, 530.
22) Furuhashi, Jap. J. med. Sci. Trans. II. Biochem., 1938, **3**, 243.
23) Sato, J. Chosen Med. Ass., 1938, **28**, 265.
24) Bogert & Plass, J. Biol. Chem., 1923, **56**, 297.
25) Kihara, Ketsueki-Gaku Togikai Hokoku (Jap.) (III), 1950, p. 118.
26) Tatsumoto, San-Fujinka no Shinpo (Jap.), 1955, **7**, 83.

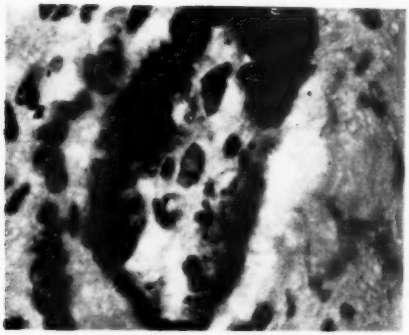


Fig 1. Peripheral villus of full-term human placenta. Incubation in $CuSO_4$ solution for 20 min. Accumulation at the part of basement membrane and net-work picture in the stroma, are visible. Objective 40×Ocular 10.

Fig. 2. Human placenta aged 3 months. Incubation for 20 min. in $CaCl_2$ solution. Calcium granules are found on the surface of syncytium cell with moss-like accumulation and in the syncytium cytoplasm, but no positive granules are found in the Langhans cells and the stroma. 40×10.

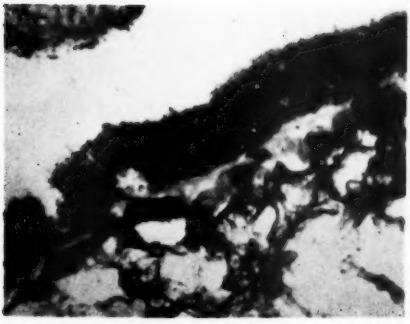


Fig. 3. Same placenta showed in Fig. 2. Incubation for one hour in $CaCl_2$ solution. Calcium granules are found in the syncytium as well as in Fig. 2. The Langhans layer is quite negative, but remarked accumulation at the part of basement membrane, and from it clear net-work stretching into the stroma is seen. 40×10.

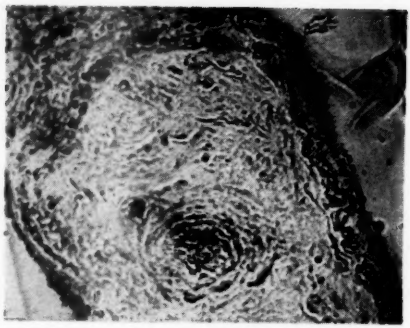


Fig. 4. Full-term human placenta. Incubation in Fatgen with bile for one hour. Fat granules are moss-wise on the surface of syncytium epithelium, and linear at the part of basement membrane. In the stroma, reticular picture is indefinite. 40×5.

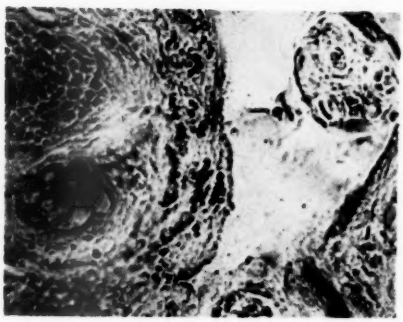


Fig. 5. Full-term human placenta. Perfusion through umbilical artery with Fatgen-bile solution. Similar pictures are seen as showed in Fig. 4 except moss-wise accumulation on the epithelial surface. 40×5.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Studies on Leucocytosis and Leucopenia in Infectious Diseases

Report IV

On the Diphasis of Mitosis of the Nucleated Cells in the Bone Marrows in Infectious Diseases

By

Akira Saito
(斎藤　章)

From the Medical Clinic of Prof. T. Kurokawa
Tohoku University Sendai

(Received for publication, February 26, 1958)

As discussed in the first–third reports[1),2),3)], the essential difference between leucocytosis and leucopenia based upon the mitotic function in the bone marrow, is that in leucocytosis the function is frankly accelerated, while in leucopenia it is frankly lowered. Concerning the genetic mechanism of leucocytosis and leucopenia Naegeli's[4)] opinion has been widely adopted. He concluded, leucocytosis was the expression of sufficiency and leucopenia was that of insufficiency of the bone marrow function. But Schottmüller[5)] expressed his opinion from the standpoint of clinical medicine that there were some points to be reconsidered in Naegeli's opinion. Fujimori[6)] held the opposite opinion to the Naegeli's and pointed out as the chief cause of leucopenia a disturbance in the maturation and the outflow into the circulating blood of the white blood cells. Heilmeyer[7)] states that the Naegeli's opinion is contradicted by the fact that in leucopenia of typhoid cases leucocytosis takes place soon after the appearance of complications such as intestinal bleedings or suppurations.

In this report the author would like to emphasize the diphasis of mitosis in the bone marrows as the most important cause of the difference between leucocytosis and leucopenia.

On the Genetic Mechanism of Leucocytosis and Leucopenia

In infectious diseases human body reacts upon the pathogenetic agents with an increase or decrease in the white blood cells in order to defend himself against them. As long as a case of the disease takes a favourable course without complications an increase or decrease in the leucocyte counts is specific for the disease. The infectious diseases in which leucocytosis appears are as follows: Various kinds of pneumonia,

meningitis and encephalitis, Asiatic cholera, rabies, smallpox, pertussis, erysipelas, diphtheria, eruptive typhus, sepsis, syphilis, tonsillitis, lymphadenitis submaxillaris, relapsing fever, Weil's disease and so forth, and purulent diseases in other departments, such as otitis media, appendicitis, liver abscess, puerperal fever etc.. The infectious diseases with leucopenia are as follows: typhoid fever, paratyphoid A, B, K (Salmonellosis Sendai), measles, rubeola, poliomyelitis, influenza, Japanese river fever, Bang's disease, Malta fever, kala-azar, mumps, erythema subitum, dengue, pappataci-fever, hepatitis epidemica, Ezo-fever (a rickettsiosis prevailing in Hokkaido), Sititô-fever (a rickettsiosis prevailing in Izu-Sititô (Izu Is.) and in the district near Mt. Fuji) etc..

On the basis of the first[1] and the third reports[3] pointing to *the leucopoiesis in leucocytosis* our opinion about it can be schematically illustrated in Fig. 1. The impulse of the pathogenetic agents reaching the bone

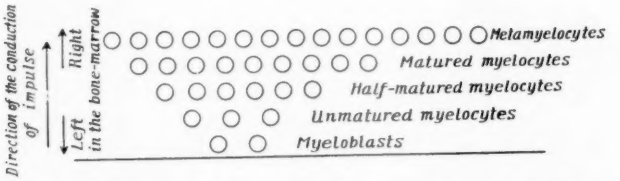


Fig. 1. The direction of the conduction of impulse to raise or lower mitosis of the nucleated cells in the bone marrow.

marrow, the primitive cells on the left of the bone marrow are first reacted and their mitosis is accelerated increasing the cells, a rise of mitosis being first seen in primitive cells and later gradually in more mature ones. As shown in Fig. 2, an increase of cells appeared successively in the variously matured cells from the left to the right in the bone marrow.

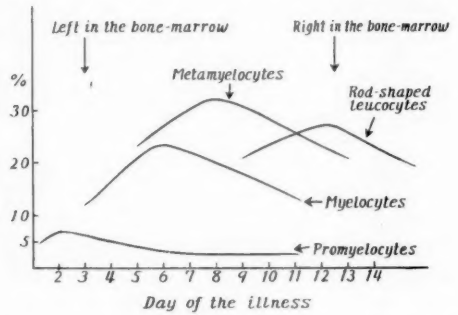


Fig. 2. The trend toward increasing the neutrophil cells in the bone marrow.

In the early stage of the illness the nearly matured cells which were on the right in the bone marrow and ready for flowing, flowed out of the bone marrow into the blood in order to comply with the peripheral demand, and consequently symptomatic leucocytosis and a nuclear shift to the left appeared in the blood, at the same time a decrease of matured cells and a symptomatic nuclear shift to the left being seen in the bone marrows. In the meanwhile mitosis of the cells on the left in the bone marrow (primitive cells) rose causing increase of the cells, showing a nuclear shift to the left. After that a rise of mitosis accompanying an increase of cells shifted gradually from the left to the right in the bone marrow. In the peak stage of the illness the mitosis index in the bone marrow reached the highest of 3.28‰, and that in the matured myelocytes rose so remarkably that the metamyelocytes, mononuclear and polynuclear leucocytes increased strikingly, showing a nuclear right shift in the bone marrow. Consequently these leucocytes actively flowed out from the bone marrow into the blood, meeting the demand and destruction of leucocytes in the periphery and showing leucocytosis with a nuclear shift to the left in the peripheral blood, but still they were abundant in the bone marrow. Further in the defervescent stage the demand and the destruction in the periphery gradually lowered and mitosis in the bone marrow dropped with a decrease of the nucleated cells. Here it is a noteworthy fact that the mitosis index showed a subnormal value of 2.30‰ in the defervescent stage, because a drop of mitosis of the nucleated cells was followed by a decrease of the nucleated cells considerably later.

$$\text{M.I. } (‰) = \frac{\text{Mitosis count}}{\text{Nucleated cell count}} \times 1{,}000.$$

In this formula the mitosis index is supernormal in the course of increase and is subnormal in that of decrease of the nucleated cells. In the reconvalescent stage the demand and the destruction in the periphery decreased, and accordingly the mitosis index showed about the normal value. After the reconvalescence a retardation of mitosis followed its acceleration in the bone marrow, showing a nuclear left shift in the bone marrow and a nuclear right shift in the peripheral blood. Accordingly the pictures of the bone marrow and the peripheral blood showed a reversed relationship, as Naegeli[8] called the blood picture the reflected image of the bone marrow picture, and both in the bone marrow and the blood frequently appeared overripened neutrophil and eosinophil leucocytes.

The leucopoiesis in leucopenia can be discussed here on the basis of our second[2] and third reports.[3] At the onset of the disease the nearly matured cells in the bone marrow flowed out of the bone marrow into the blood to comply with the demand from the periphery. Consequently a decrease

of the nucleated cells and a symptomatic nuclear shift to the left appeared in the bone marrow, at the same time as a symptomatic leucocytosis and a nuclear shift to the left appeared in the peripheral blood. These changes are similar to those seen in leucocytosis. After that a drop in mitosis of the nucleated cells appeared first in the primitive cells on the left in the bone marrow, but a nuclear shift to the left was observed as before, because of a remarkable decrease of the matured cells in the bone marrow owing to their accelerated outflow of the matured cells into the blood.

Then mitosis dropped more and more, shifting the drop of mitosis toward the right in the bone marrow, while the peripheral demand and destruction were accelerated gradually in the course of the illness, and the nearly matured cells flowed out actively from the bone marrow into the blood, but the peripheral demand could not be filled. Consequently leucopenia became gradually remarkable in the blood, and the decrease of the nucleated cells with a nuclear left shift became strikingly conspicuous in the bone marrow. In the bone marrow the mitosis index showed the minimal value of 1.94‰ and a nuclear shift to the left became most remarkable in the peak stage, the nucleated cell count showing the minimal value in the latter half of the peak stage and the defervescent stage. Thereupon Barta[9] termed the bone marrow in the fever-rising stage "a metamyelocytic marrow", that in the peak stage and the defervescent stage "a myelocytic, rarely myeloblastic marrow", Henning[10] called that "a strongly left shifted one" and Storti & Filippi [1] named it "a cell-poor marrow".

It is a noteworthy fact that in the peak stage, when rod-shaped and segmented leucocytes decreased remarkably, the percentages of the lymphocytes were strikingly elevated in the bone marrow as those seen in the blood.

The trend toward decreasing the nucleated cells in the bone marrow was projected on the peripheral blood and during the peak stage and defervescent stage a most remarkable leucopenia with a relative lymphocytosis and a remarkable nuclear shift to the left appeared, frequently showing an extreme leucopenia of 1,000–2,000 white blood cells in the blood. In the defervescent stage mitosis showed a trend toward rising while the mitosis index showed a supernormal value of 2.52‰ because of the remarkable decrease of the nucleated cells in the bone marrow. The reason can be readily understood in reference to the above-mentioned formula. A rise in mitosis appeared first in the primitive cells and later in more mature ones. In the reconvalescent stage mitosis rose more, showing about the normal value of 2.26‰. A rise in mitosis appeared first in the unmatured and the half-matured myelocytes, and after that the rise in mitosis shifted to the right in the bone marrow, attaining the normal level.

This is the reason why a nuclear left shift was apparent and overripened nutrophil and eosinophil leucocytes could not be seen both in the bone marrow and the blood in the recovalescence of leucopenia cases, even when the leucocytes had increased to the normal level in the peripheral blood. In general it may be said, the duration of rise or drop of mitosis is proportionate to the clinical course of the diseases: in a prolonged course of the illness, such as in severe typhoid fever, the drop of mitosis is prolonged remarkably, showing leucopenia with a nuclear left shift in the blood even in the third week after the termination of fever, but in the case of paratyphoid B it is shortened relatively.

The Maturation of the Nucleated Cells in the Bone Marrow

As a cause of leucopenia Fujimori[6] has pointed out a disturbance in the maturation of the nucleated cells in the bone marrow of typhoid fever. He took a nuclear shift to the left both in the bone marrow and the blood as a proof of his opinion, but the cause of the difference between leucocytosis and leucopenia does not consist in the maturation of the cells, for leucocytosis and leucopenia are not classified from the standpoint of the maturation of cells, but from that of the white blood cell count in the blood. Of course, it is usual that some disturbance in the maturation of cells, such as toxic granules, is observed in the cases of infectious diseases, but it can be seen not only in the case of leucopenia but also in that of leucocytosis. Accordingly it is evident that changes of the leucocyte count in the blood depend upon mitosis in the bone marrow.

The Outflow of the White Blood Cells from the Bone Marrow into the Blood

The rapidity of the outflow is, as Rohr[12] has stated, not controlled by the bone marrow itself, but dependent on whether in comparison with the normal the falling gradient of a difinite kind of cells between the bone marrow and the blood is greater or lesser. This potential falling gradient is mostly influenced from the peripheral side; it is enlarged by increase of destruction, and lessened by decrease of demand in the periphery. It can be also changed by a rise or drop of the mitotic function in the bone marrow. The greater is the falling gradient, the more rapidly and plentifully flow out the younger cells of the bone marrow, showing a remarkable nuclear shift to the left in the blood. Furthermore, both in leucocytosis and leucopenia the more suddenly and seriously the onset of the illness occurs, the greater is the falling gradient since the peripheral demand and destruction increase strikingly from the very onset of the illness, and consequently in such cases many younger cells, such as oxyphil normoblasts,

matured myelocytes and metamylocytes, appear in the peripheral blood. The falling gradient is greatest at the onset of the illness and during the fever-rising stage, after that lessening gradually to return to the normal. Thus the mechanism of the outflow of the white blood cells into the blood can be easily understood in leucocytosis as Fig. 3 and 4 show, but that in leucopenia comes into question. Galinowski[13] and Fujimori[6] have pointed

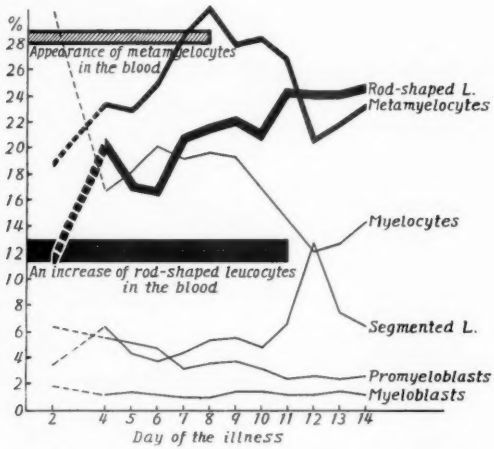


Fig. 3. Change and outflow of various kinds of the nucleated cells in the leucopoietic system in the bone marrow of leucocytosis cases.

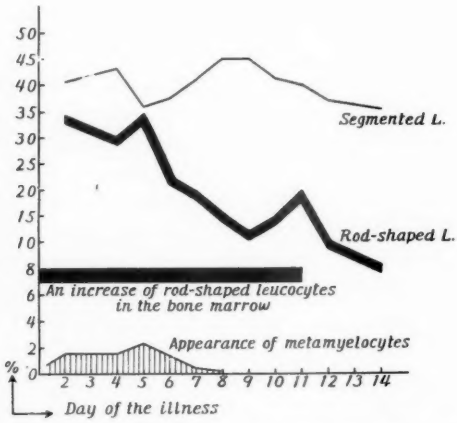


Fig. 4. Appearance and increase of the younger cells in the peripheral blood of leucocytosis cases.

out a disturbance in the outflow of the white blood cells from the bone marrow into the blood as a cause of leucopenia. In the peak stage of leucopenia the nucleated cells, particularly matured cells have decreased in the bone marrow and simultaneously the white blood cells have diminished equally in the blood, consequently the falling gradient have lessened so strikingly that it is a matter of course that few of the white blood cells can flow out of the bone marrow into the peripheral blood as Fig. 5 and 6 show. That is,

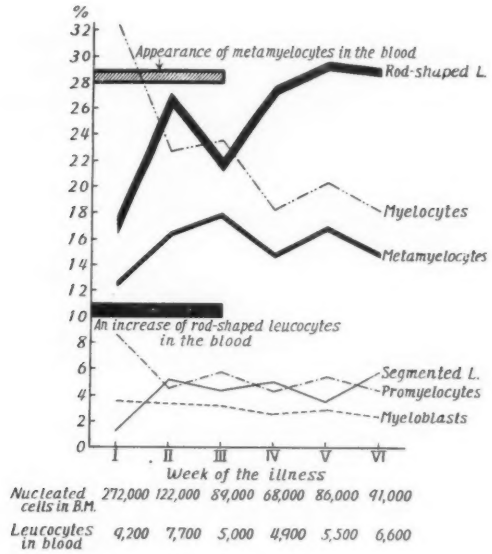


Fig. 5. Change and outflow of various kinds of the nucleated cells in the leucopoietic system in the bone marrow of leucopenia cases.

in leucocytosis the falling gradient lessens in the course of the illness in a large count, while in leucopenia it lessens gradually in a small count of the white blood cells, attaining the normal value in the reconvalescence.

Here it is a noteworthy fact that the younger cells appeared or increased in the peripheral blood in the case of leucocytosis as long as they were increasing in the bone marrow (Fig. 3), and in the case of leucopenia as long as the white blood cells were decreasing in the peripheral blood (Fig. 6). Indeed, in the autopsy of a case with leucopenia such as typhoid fever many necrotic foci are seen in the myeloid tissues, as many authors have reported, but in typhoid fever such foci are to be seen in other parts of the entire body as often, and what is more, these findings are seen not only in leucopenia but also in leucocytosis such as in sepsis etc. In short

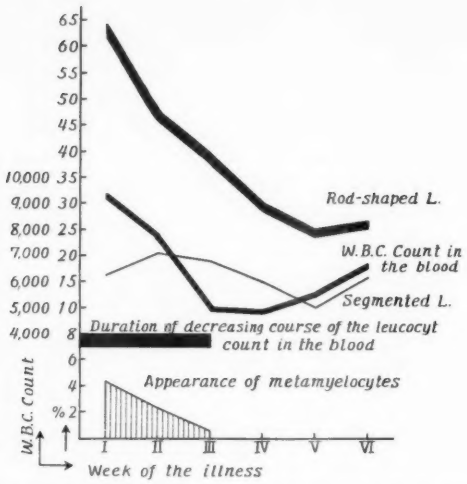


Fig. 6. Appearance and increase of the younger cells in the peripheral blood of leucopenia cases.

there is not much disturbance in the outflow of the white blood cells as to be accounted as a cause of leucopenia.

The Destruction of White Blood Cells in the Periphery

Concerning the genetic cause of leucopenia Faludi[14] has pointed out the destruction of white blood cells. That is, the decrease of the leucocyte count is principally caused by a destruction of the leucocytes within the blood vessels. Rohr[12] has stated his opinion in his work," It is probable that leucopenia can take place partly in consequence of the destruction of the white blood cells within the blood vessels in spite of accelerated new formation ".

If Faludi's[14] opinion can be true, the mitosis index in leucopenia must be greater than that in normal person, for in the above-mentioned formula of the mitosis index the nucleated cell count in the bone marrow of leucopenia is lessened.

However in our examination the mitosis index in leucopenia is contrary smaller than the normal value. So the author can't support Fauldi's opinion.

In our examinations in the cases of typhoid fever the destruction increased gradually from the onset through the entire course of the illness, showing the maximal height at about the peak stage, but such an increase was observed not only in leucopenia but also in leucocytosis and there is

no evidence that the destruction of white blood cells in leucopenia is more accelerated than in leucocytosis. Both in leucocytosis and leucopenia degenerative pictures such as degeneration of nuclei, formation of vacuoles in the nuclei and the protoplasm of the white blood cells appeared in the bone marrow and the blood, particularly in the peak stage. These pictures appeared first in the cells on the left of the bone marrow and in the course of the illness gradually in the cells on its right. Thereafter, they were seen in the peripheral blood, appearing first in the cells on the left and later on in those on the right of the peripheral blood. It is a noteworthy fact that these degenerative pictures were seen still in the bone marrow after they had disappeared from the peripheral blood.

As mentioned above in leucocytosis and leucopenia in infectious diseases there is not much difference in the maturation, the outflow into the circulating blood and the destruction of the white blood cells, but a perceptible inequality is observed in their mitotic function; in leucocytosis it is frankly accentuated, while in leucopenia it is equally frankly lowered. The rise and drop of mitosis are proportionate to the severity of the illnesses and the duration of their courses.

On changes of the lymphocytes in infectious diseases, both in the bone marrow and the peripheral blood the lymphocytes decreased relatively in leucocytosis while they increased relatively in leucopenia. Regarding the participation of the spleen in the change of leucocytes, we may point out that enlargement of the spleen is observed in leucopenia such as typhoid fever, paratyphoid A, B and K, Japanese river fever, kala-azar and in leucocytosis such as sepsis, eruptive typhus, recurrent fever and so on. So leucocytosis and leucopenia can not be classified by enlargement of the spleen.

Diphasis of Mitosis of the Nucleated Cells in the Bone Marrow in Infectious Diseases

In infectious diseases the human body reacts sensitively against pathogenetic microbes and their toxins with fever. Of his defense mechanism the white blood cells are the foremost and the most active. Accordingly the qualitative and quantitative changes of the white blood cells are specific to a definite kind of infectious diseases. It is usual in infectious diseases that a probable and not rarely sure diagnosis can be established on the basis of the changes of the white blood cells in the blood. According to the white blood cell count, all the infectious diseases can be divided into 2 groups: leucocytosis and leucopenia. From the angle of the genetic mechanism, the difference between the two is seen in their mitotic function in the bone marrow; in leucocytosis it is frankly accelerated while in leucopenia it is frankly lowered. Fig. 7 shows a graphic description of

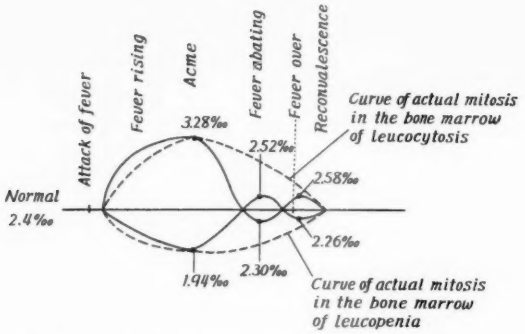


Fig. 7. Curves of mitosis index of the leucopoietic system in the bone marrow in the course of leucocytosis and leucopenia.

mitosis index of the leucopoietic system in the bone marrows in the course of leucocytosis and leucopenia. Taking the trend of normal mitosis index of 2.4‰ as the axis of coordinates, values of mitosis index in every stage of leucocytosis and leucopenia are fixed as point coordinates; 3.28‰ in the acme, 2.30‰ in the fever-abating stage and 2.58‰ in the reconvalescence of leucocytosis and in the same way 1.94‰ in the acme, 2.52‰ in the fever-abating stage and 2.26‰ in the reconvalescence of leucopenia. These points traced successively represent a pair of wavy curves.

Furthermore when these curves are corrected on the basis of outflow of leucocytes into the peripheral blood and changes of the nucleated cell counts in the bone marrows, they can be shown as a pair of simple symmetric curves with trends toward rising and dropping respectively (dotted curves), indicating the actual mitosis of the leucopoietic system in the bone marrows of leucocytosis and leucopenia. The author has named the mitotic course with trend toward rising the first phase, that with trend toward dropping the second phase, and factors of the former the first-phase factors, those of the latter the second-phase factors. Each of these factors has its specificity.

In infectious diseases at the onset of the illness first the leucocytes stored in the bone marrow flow out into the blood in order to fill up the peripheral demands both in leucocytosis and leucopenia, and consequently a nuclear shift to the left was seen both in the bone marrow and the blood. Then the bone marrow is irritated by impulse of the pathogenetic microbes and reacts with a rise or drop of mitosis according to the specificity of their factors, the rise or drop of mitosis beginning in the primitive cells and appearing later in the more mature ones. In the bone marrow a rise of mitosis shows an increase of the nucleated cells with a nuclear left shift,

while a drop of mitosis shows a decrease of those with a relative nuclear left shift in consequence of the outflow of the matured cells in the fever-rising stage. After that in the diseases of the first-phase type a new proliferation actively takes place in the more matured cells, consequently a nuclear right shift appears owing to a rise of mitosis of the more matured cells in the bone marrow, but on the contrary in the diseases of the second-phase type a decrease of the nucleated cells appears in the more matured cells and a nuclear left shift is obvious in the bone marrow in consequence of the outflow of the matured cells into the blood. Consequently in the peak stage of the first-phase type the increase of the nucleated cells, particularly the matured cells with a nuclear right shift in the bone marrow, is very conspicuous, showing leucocytosis with a nuclear left shift in the peripheral blood, while on the other hand in the peak stage of the second-phase type it is very remarkable that a decrease of the nucleated cells, particularly the matured cells with a nuclear left shift in the bone marrow and leucopenia with a nuclear left shift in the blood is observed. After the fever-abating stage the mitotic function becomes lower with a decrease of the cells in the first-phase type, but it is accelerated with an increase of the cells in the second-phase type, attaining the normal level in the reconvalescence.

Thus both in the first- and the second-phase types the normal bone marrow is irritated by impulse of the pathogenetic microbes and reacts physiologically against them according to the specificity of their factors. Therefore it is a mistake to take thoughtlessly an increase of the cells for sufficiency and a decrease of those for insufficiency of the bone marrow. It is understood without difficulty that leucopenia in infectious diseases is not similar to that seen in severe pneumonia, perityphlitis, sepsis and such diseases, in which instead of leucocytosis observed as usual, a decrease of the white blood cells takes place because of insufficiency of the bone marrow. An insufficiency of the bone marrow can be recognized when in the course of leucopenia such as typhoid fever the white blood cells remain stationary on a low level and do not show the slightest increase notwithstanding severe pneumonia, intestinal perforation or other complications.

The fact mentioned above can be easily understood by the following Figure. Fig. 8 shows the irritability of the bone marrow. The irritation of the bone marrow follows the attack of fever, somewhat (1–2 days) tardily, and rises gradually in the course of the illness, attaining the maximal height in about the acme stage. After the defervescent stage it lowers gradually, returning to the normal in the reconvalescence. Therefore the curve of irritability of the marrow runs parallel to the curve of fever with some lag. The irritation of the bone marrow takes place in the form of

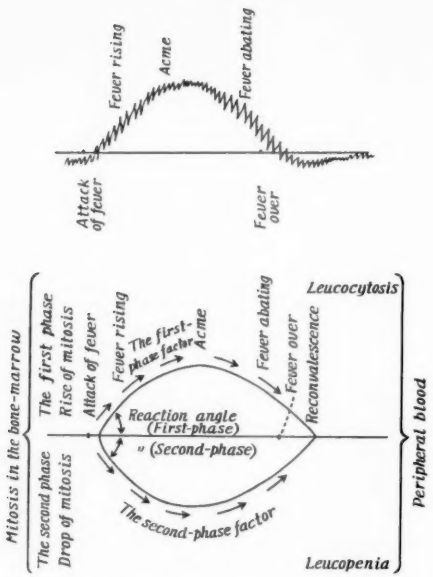


Fig. 8. Schemas of the irritability to stimulus and of the reaction mechanism of the bone marrow.

a rise of mitosis in the bone marrow of the first-phase type, and of a drop of mitosis of the second-phase type. As shown in Fig. 8, the course of mitosis is illustrated as a curve of rising in the former and as that of dropping in the latter. Call as the reaction angle the angle which is made by a normal trend of mitosis and a trend toward increase or decrease of mitosis, and the larger the reaction angle, the greater is the increase or decrease of the nucleated cells. The reaction angle is proportionate to the specificity, the quality and the quantity of the factor of the microbes and the disposition of individuals. The toxins of the pathogenetic microbes causing leucocytosis belong to the first-phase factors and those causing leucopenia to the second-phase factors. In the case more than two factors are added up, if these factors belong to the same phase, the curve of mitosis elevates or drops more strikingly according to a summation of the factors in the same phase, while if the factors belong to different phases the curve of mitosis changes its trend toward rising or dropping according to the difference of the factors.

These relations are illustrated in Fig. 9. For instance, in a typhoid fever case complicated with pneumonia or intestinal perforation, if the first-phase factor is greater than the second-phase factor, the trend of

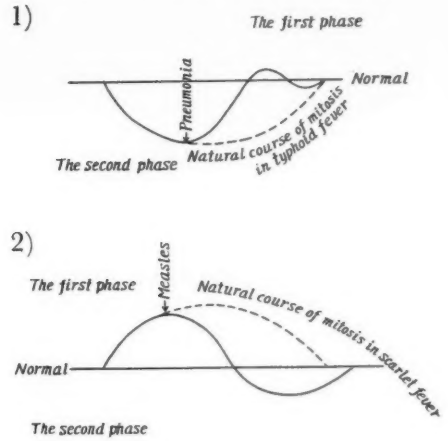


Fig. 9. 1) Case in the second phase, in which in the course of the illness a second-phase facter is complicated with a first-phase factor (typhoid fever case complicated with pneumonia).

2) Case in the first phase, in which vice versa in the course of the illness a first-phase factor is complicated with a Second-phase factor (scarlet fever case complicated with measles).

mitosis changes toward rising and the curve of mitosis changes equally as shown in Fig. 9–1), showing an increase of the white blood cells in the peripheral blood according to the difference of the first and the second phase factors. Fig 9–2) shows the curve vice versa of a scarlet fever case complicated with measles.

Here the specificity of factors comes into the question. The factors, even belonging to the same phase, have not only biologically but also clinically all their respective specificity. For example, the pathogenetic microbes of typhoid, paratyphoid K, A and B are called the germs of the typhoid group in our country and belong to the second-phase factors, but they have all their respective specificity as follows.

Biological specificity

1) In cultivation. The colonies of typhoid bacilli are the thinnest and dewy, those of paratyphoid K and A bacilli somewhat thicker and those of paratyphoid B bacilli are the thickest and opaque like those of coli bacilli. 2) In sugar-decomposition. The ability of sugar-decomposition is the lowest in typhoid bacilli, moderate in paratyphoid K and A bacilli, the greatest in paratyphoid B bacilli. 3) Production of immune bodies. The immune bodies seem to be produced most actively in typhoid. 4) Antibiotic sensitivity. Typhoid bacilli are most sensitive

to antibiotics such as chloromycetin, followed by paratyphoid K, A and B in the order named. In our examinations the dosage of chloromycetin was 0.25 mg. every three hours for seven days. Fever disappeared within 2–5 days in typhoid cases, within 4–9 days in paratyphoid A cases, but continued longer in paratyphoid B cases and in some of these cases the usefulness of chloromycetin could be scarcely recognized.

Clinical specificity

1) Severity of the illness. In general typhoid fever is severe, paratyphoid K and A moderately severe but paratyphoid B is mild. 2. Duration of the fever stage. Fever continues for the longest time in typhoid, for a moderate time in paratyphoid K and A, but only for a short time in paratyphoid B. 3) Existence of the bacilli in the bone marrow and blood was observed by Saito[15]. The typhoid bacilli exist in the bone marrow and blood for the longest time, paratyphoid K and A bacilli for a moderate time and paratyphoid B bacilli only for a short time. 4) The blood sedimentation rate. The blood sedimentation rate is either normal or only slightly elevated at the onset of the illness but after that it shows a decided increase and reaches its maximum at about the time of the termination of fever. It increases most remarkably in typhoid, moderately in paratyphoid K and A, but only slightly in paratyphoid B. 5) Anemia appears in proportion to the severity and duration of the illness. Accordingly it is the most remarkable in typhoid, moderate in paratyphoid K and A, but only slight in paratyphoid B. 6) Leucopenia appears most strikingly in typhoid, moderately in paratyphoid K and A, but slightly in paratyphoid B. 7) The positive percentages of diazo-reaction, albuminuria and enlargement of spleen are the highest in typhoid, moderate in paratyphoid K and A, but only slight in paratyphoid B. As mentioned above it can be found that even the factors belonging to the same phase all have orderly and definitely the respective specificity and that the reaction of the pathogenetic microbes against the human body is the most active in typhoid bacilli, followed by paratyphoid K, A and B in the order named. The reaction angles of 4 of the second-phase factors are in the order of typhoid>paratyphoid K>paratyphoid A>paratyphoid B. From these reasons it can be easily understood that in the peripheral blood leucopenia is the most striking in typhoid, moderate in paratyphoid K and A and slight in paratyphoid B. In the same way, among rickettsioses such as Japanese river fever, Ezo-fever and Sititô-fever the reaction is the most remarkable in Japanese river fever, moderate in Ezo-fever and slight in Sititô-fever.

Saito and his coworker Koizumi[16] analysed the bodily components of the typhoid bacilli into three fractions: mucoid fraction, protein fraction and polysaccharide fraction. We found that the mucoid fraction was

the most predominant in biologic reactions.

Relation Between the two Phase Factors and the Autonomic Nerve System

In my opinion concerning the relation between the two phase factors and the autonomic nerve system, the first-phase factor causes sympathicotonia with a rise of mitosis and the second-phase factor vagotonia with a drop of mitosis.

Conclusion

The author has studied the genetic mechanism of leucocytosis and leucopenia in various infectious diseases. In our systematic serial examination of the hematopoietic function of the bone marrow in the course of the diseases, the author has found that the difference between leucocytosis and leucopenia originates from a diphasis of mitosis in the bone marrow.

References

1) Saito & Ono, Tohoku J. Exp. Med., 1958, **67**, 253.
2) Saito & Shimoyama, *ibid.*, 1958, **67**, 265.
3) Saito & Miyamoto, *ibid.*, 1958, **67**, 341.
4) Naegeli, Blutkrankheiten u. Blutdiagnostik, Julius Springer, Berlin, 1931, 236, 239, 589.
5) Schottmüller, Handb. inn. Med., 1838, 2 Ed., **1**.
6) Fujimori, Hokkaido Igaku Zassi (Jap.), 1938, **16**, 1, 897.
7) Heilmeyer & Begemann, Handb. inn. Med., 11 Bd., Blut u. Blutkrankheiten, Julius Springer, Berlin, 1951, 535.
8) Naegeli, Cited by Rohr, Das menschliche Knochenmark, Georg Thieme, Leipzig, 1940, 171.
9) Barta, Folia haemat., 1933, **50**, 287.
10) Henning, Dtsch. med. Wschr., 1935, 1543.
11) Storti & Filippi, Sang, 1937, **11**, 440.
12) Rohr, Das menschliche Knochenmark, Georg Thieme, Leipzig, 1940, 171.
13) Galinowski, Folia haemat., 1938, **60**, 381.
14) Faludi, *ibid.*, 1938, **59**, 357.
15) Saito, Tohoku J. Exp. Med., 1955, **62**, 161, 173, 183.
16) Koizumi & Saito, Tohoku Igaku Zassi (Jap.), 1942, **31**, 597.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Two Disaccharides Separated from a Probable Mixture of Dermoitinsulfuric and Chondroitinsulfuric Acids— Preliminary Note

Mei Satake and **Hajime Masamune**
(佐 武 明) (正 宗 一)

The Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, June 10, 1958)

We have separated two disaccharides from a probable mixture of sodium dermoitinsulfate (Aizawa)[1] and sodium chondroitinsulfate in the proportion of about 4:1. The material was prepared from pig skin and proved homogeneous electrophoretically. $[\alpha]_D$ (water) -64.8°; N, 2.6; hexosamine, 30.5; hexuronic acid as glucuronic acid, 46.5 (orcinol) or 12.9 (carbazole); S, 5.5; acetyl, 7.0; ash (Pregl), 22.8. The acid hydrolysate gave two distinct spots with Rf values of L-iduronic acid and of its lactone but only a faint spot with an Rf value of glucuronic acid after paper-chromatography.

Next, the material was changed into the barium salt, and 1.1 g. of this salt was hydrolyzed with 0.5 *N* H_2SO_4 (50 cc.) (6 h., 100°), and freed from sulfuric acid with barium carbonate and baryta. The clear fluid given (pH 4.2) was chromatographed on a 1.4×12 cm. column of Dowex 50 (hydrogen form) with water at first and then with acetic acid solutions of increasing concentrations. The fractions of 0.005 *N* acetic acid eluate showed two peaks of orcinol reaction and those showing the first peak gave 34 mg. of crystals in needles (**I**) on concentration. The fractions showing the second peak were condensed and lyophilyzed, and the amorphous powder gave 59 mg. of colorless crystals (long whetstones) (**II**) from water (Fig. 1). After ninhydrin oxida-

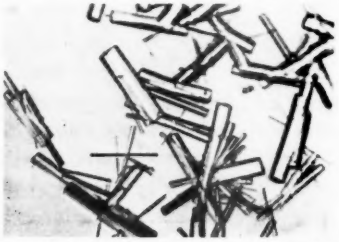


Fig. 1. Microphotograph of crystals of disaccharide **II**.

tion with subsequent hydrolysis and paper chromatography, **I** showed three spots at the positions corresponding to lyxose, glucuronic acid and glucuronolactone, suggesting chondrosine, and **II** spots at the positions of lyxose, L-iduronic acid and L-iduronolactone. **II** recrystallized from water: F.P., 170° (decomp.); $[\alpha]_D$ (water) +39.7°; N, 3.61; C, 36.84; H, 6.34; hexosamine, 41.0; hexuronic acid as glucuronic acid, 58.5 (orcinol) or 13.6 (carbazole); iodine use as glucose (Macleod-Robison), 103.9*; crystal water, 2 molecules. The infrared spectrum and the fact that it gave a neutral solution in water excluded γ-lactone ring. The lyxose-uronide obtained by oxidation of it with ninhydrin gave the negative result when tested with alkaline triphenyltetrazolium, namely, it was revealed that, in **II**, C-3 of galactosamine is bound by the uronic acid glycosidically.

References: 1) Aizawa, Tohoku J. Exp. Med., 1957, **65**, 375.

*The corresponding value of chondrosine 104.4.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

On the Transportation Channels of Substances in the Stroma of Placental Villi

By

Kiichiro Noda, Yujiro Yamada, Tsuneo Murakami
(野田 起一郎) (山田 雄二郎) (村上 恒男)
and **Shoichi Itagaki**
(板垣 昭一)

From the Department of Obstetrics and Gynecology, Faculty of Medicine, Tohoku University, Sendai;
Director: Prof. K. Kushima

(Received for publication, March 1, 1958)

Works in the past upon the details of the mechanism of substance exchange in the placenta have been regrettably few, the mechanism being generally interpreted only in a schematic way in analogy with the process of gas exchange in the lung. There is, however, a wide anatomical difference between the placenta and the lung, the exchange of matters between the fetal blood and the maternal blood in the placenta being effected through the epithelium and the stroma of the villi and the walls of the capillaries. So, we can readily surmise the existence of some mechanism or channel of matter transportation in this part, but the past studies have been limited to the researches on the epithelium of the villi and little, if any, research has been conducted on the villous stroma and blood capilaries.

At this Department, we have been studying on the metabolism and the permeability of the placenta, availing ourselves of the histochemical technics rapidly advanced in recent years. Several reports[1),2),3),4)] have been already published on the results of these studies, and the present authors will report hereunder on the interesting findings suggestive of the transportation channels of matters in the stroma of the placental villi.

Experimental

Materials and Methods

The materials used were human placentae obtained at term, and with copper, iron, calcium and glucose, we made the three series of experiments described below, then sections were sampled choriodecidually from the central and the marginal parts of the placenta and stained for microscopic examination, as given below.

1) Injection through the maternal cubital vein (Group M). Copper was used in the form of copper sulphate, 20 cc. of its 0.5% isotonic solution being mixed with 200 cc. of isotonic saline for slow intravenous injection. For iron administration a mixture of 15 cc. of 1% iron gluconate solution and 40 cc. of 20% glucose solution was used and calcium was injected in 40 cc. of 2% calcium chloride solution, while 100 cc. of 50% glucose solution was used in the glucose administration tests. The placental specimens were sampled upon parturition taking place immediately following or within 12 hours after the injection.

2) Injection through the umbilical artery (Group U). 200 cc. of the copper sulphate solution above, 200 cc. of a 0.03% iron gluconate solution in isotonic saline, 300 cc. of a calcium chloride solution in isotonic saline containing calcium in the concentration of 100 mg/dl or 200 cc. of a 5 % glucose solution was injected into the placenta through the umbilical artery immediately after delivary of placenta.

3) Incubation (Group I). The decidual membrane was carefully peeled off the placenta immediately after expulsion and the placenta was incubated for 5–30 minutes at 37°C in isotonic copper sulphate, iron gluconate, calcium chloride or glucose solution.

Staining

For histochemical detection of copper, iron, calcium or glucose, Okamoto-Utamura's paradimethyl-aminobenzylidenrhodanine staining[5] and rubeanic acid staining, Prussian blue and Turnbull's blue staining, Yamashita's variation[4] of Kossa's method, or Okamoto-Kadoto-Aoyama's method[6] of using toluidine blue staining in combination were respectively applied to stain the sections concerned. The original Bielschowsky's method, a variation of it by Foot or Gomori was used for impregnating argyrophilic fibrils.

Results

After copper administration, in the placentae from mothers subjected to the injection(Group M) no finding was observed different from the untreated placentae, but in the incubated specimens (Group I), a diffuse copper reaction was already observed in the syncytium cells of the villi and copper granules were found arranged linearly along the basement membrane beneath these cells. These copper granules stretched in lines further into the stroma and forming a net-work by mutual anastomosis, partially reached the walls of capillaries. In the placentae injected with copper sulfate solution through the umbilical cord (Group U) too, such reticules of copper granules stretching from the capillary walls into the stroma were observed and copper granules were detected also in a part of the basement membrane, but the result of searching for them in the syncytium cells proved negative. Such findings were observed only in a part of peripheral villi with normal epithelium, while in the villi of which the epitheliar layer had disappeared and the stroma directly touched the fibrinoid tissue, a quantity of copper was observed in the fibrinoid mass alone, but no copper could be found in the stroma.

Iron administration was followed by results nearly identical with those after

copper injection as above.

In the experiments with calcium, the Group M showed little findings different from the control specimens, as in the preceding series, but in the Group I, we found a large quantity of calcium diffused moss-wise in the surface of the syncytium cells, and a small quantity of the element was found also in the cytoplasm of syncytium cells in nearly uniform distribution. Calcium was also found aggregated in lines in a somewhat larger quantity along the basement membrane beneath the syncytium cells, and in some part stretching into the stroma in the form of fibres and nets. However, it rather accumulated much in the fibrinoid tissue. In the Group U, a net-work of calcium was found running along the fibres stretched from the capillaries into the stroma and linear aggregation of calcium was observed beneath the epithelium of the villi, but the calcium granules in the cytoplasm of the syncytium cells were very small in number.

In the experiments of glucose administration, no appreciable difference was observed in the Group M, except in the placenta expelled 30 minutes after the administration, which contained glucose in its villous epithelium. In the Group I, glucose granules were found arranged in a line beneath of the syncytium cells and forming a net-work between the same part and the capillary walls. The granules of glucose were absent in the stroma of the villi covered by fibrinoids and devoid of epithelium, as in the copper and iron. Similar pictures were seen also in the Group U and I.

Staining of argyrophilic fibrils

No argyrophilic febrils were found in the chorionic plate and the connective tissue of the stem of villi, light brown-red collagenous fibres alone being detected under Bielschowsky's original staining. Rarely, these were some parts under the epithelium in the stems of villi and branches where argyrophilic fibrils were detected, blood capillaries being found in such parts also. No collagenous fibres could be found in the villi. In thick specimens, the capillaries were represented by argyrophilic nets forming tubes (Gitterröhre). These argyrophilic fibrils extend into the stroma, forming reticules, and were found connected with the basement membrane consisting of coarse argyrophilic fibrils under the syntytium cells. In the villi lacking in epithelium and covered by fibrinoids, no argyrophilic fibrils but only collagenous fibres were observed.

Summary and Discussion

When copper, iron, calcium or glucose was injected into the maternal veins, no noteworthy finding was obtained, but in the incubation experiments, the administered substance was always found aggregated into very perceptible lines along the basement membrane of the villous epithelium and in varying degrees also in the stroma in fibrous or reticular arrangements, reaching up to the capillary walls. The fibres in the connective tissue of iron and glucose granules were nearly as fine as the argyrophilic fibrils stained out by silver impregnation, but those of copper and calcium were somewhat coarser.

In the experiments of administering these substances through the umbilical artery too, similar results were obtained, except that calcium and iron were found nearly absent in the villous epithelium.

As cited above, the authors have obtained experimental results indicating the presence of the respective substance used in incubating the placental specimens, arranged in lines and reticules. From the fact that the running courses of these lines and nets are similar to those of the argyrophilic fibrils detected by silver impregnation, that no such reticular formation has ever been proved to exist outside the stroma of the villi and that the said substances were not to be found in the parts lacking in argyrophilic fibrils, such as the part of villi under fibrinoids, we feel nearly assured in assuming the location of the administered substances within the argyrophilic fibrils in the stroma of the villi. If we accept the contention of Nageotte[7] and Doljanski-Roulet[8),9] that the argyrophilic fibrils are essentially flows of tissue fluids mainly consisting in protein in vital stage, they should not be such solidly fixed entities as we see in stained preparations, but should be more free formations, and the findings that the fibres formed by different substances are not always of the same size does not constitute any ground for disputing the above our assumption. Thence, we are led to the hypothesis that the argyrophilic nets in the stroma serve as transportation routes of substances between the epithelium and the capillaries of the villi.

The argyrophilic fibrils in the placenta were found independently by Costero[10] and Zaltan[11] in the connective tissue of the villi by Bielschowsky's silver impregnation, and these authors discribed these as collagenous fibres in a preliminary stage of development. Later, Wienbach[12] found argyrophilic fibrils in existence around the capillaries of the villi and at the same time observed that the cells of the epithelium over the parts rich in argyrophilic fibrils are denucleated and flattened, while those in the positions poor in argyrophilic fibrils contain nuclei and remain unflattened.

On the other hand, Hofbauer[13] found that the iron arranged in reticularly in the stroma of the villi, but he did not touch upon the relation between the argyrophilic fibrils and such reticules.

In Japan, Kihara[14] has made an interesting study on many organs and tissues and published the opinion that the net-work of argyrophilic fibrils serve as extravascular pathways for fluids, supplying channels for absorption and efflux to lymph capillaries and for absorption to venules. His disciple Tatsumoto[15] claims that the argyrophilic fibrils in the placental villi also have the meaning as extravascular fluid pathways.

The results obtained by the present authors as described above may be said to have given positive grounds endorsing the opinion of Tatsumoto.

Conclusion

Placentae obtained at term were incubated in solutions of copper, iron or calcium compounds or of glucose, or injected with similar solutions through the umbilical artery, and we could demonstrate the existence of reticular arrangements of the administered substances in the villous stroma in all the cases. This finding, coupled with our finding in silver-impregnated specimens, led us to conclude that the argyrophilic fibrils in the stroma of the villi can serve as transportation routes between the epithelium of the villi and the villous capillaries.

References

1) Imamura, Nippon Sanka-Fujinka Gakkai Zasshi (Jap.), in press.
2) Sugawara, *ibid.*, in press.
3) Yamashita, *ibid.*, in press.
5) Okamoto *et al.* Acta scholae med. Kioto, 1938, **20**, 573.
6) Okamoto *et al.*, Nippon Taishitsugaku Zasshi (Jap.), 1948, **14**, 35.
7) Nageotte & Guyon, Am. J. Path., 1930, **6**, 631.
8) Doljanski & Roulet, The Protoplasma, 1935, **23**, 29.
9) Roulet, Erg. d. allg. Path., 1937, **32**, 1.
10) Costero, Z. f. Anat. u. Entwickl., 1931, **96**, 766.
11) Zaltan & Szathmary, Arch. f. Gyn., 1932, **149**, 173.
12) Wienbach, Z. f. microsk. Anat. Forsch., 1936, **39**, 135.
13) Hofbauer, Hoppe-Seyler's Z. f. physiol. Chem. 1903/1904, **40**, 240.
14) Kihara, Ketsuekigaku Togikai Hokoku. (Jap.) Vol. III, 1950, p. 48.
15) Tatsumoto, San-Fujinka No Shinpo (Jap.), 1955, **7**, 83.

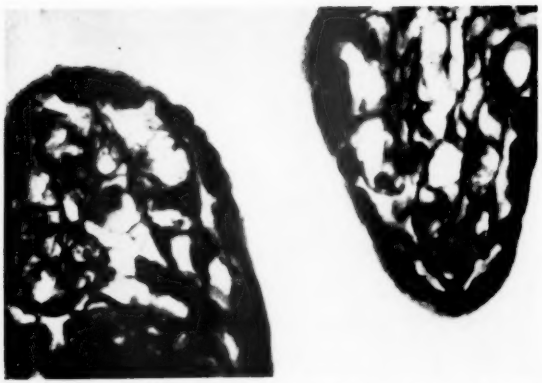


Fig. 1. Argyrophilic fibrils in the stroma of the villi. High magnification.

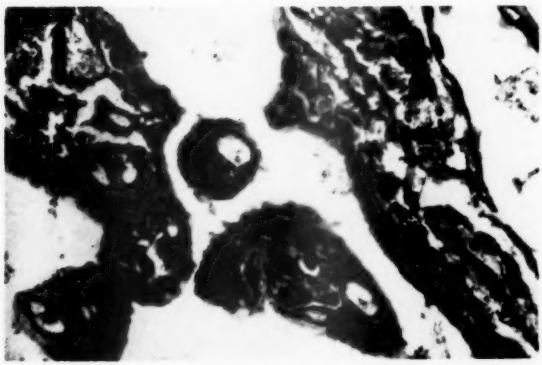


Fig. 2. Glucose in the stroma of the villi observed in the Group I. High magnification.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Effect of Oral Administration of Glucose on Blood ATP Level in Diabetic Patients

By

Yoshio Goto, Isao Ito and **Yukio Ujiie**
(後藤 由夫) (伊藤 伊三雄) (氏家 幸雄)

From the Medical Clinic of Prof. T. Kurokawa, Tohoku University School of Medicine, Sendai

(Received for publication, March 1, 1958)

In order to see the entity of metabolic disturbances in diabetes mellitus, the past experiments have been made usually on experimental diabetic animals. The results obtained by this way, however, not always reflected the essential and specific disturbance of clinical diabetes, but sometimes such side disturbances as ketosis, etc. We believe that it is more convenient and partinent to observe the changes in the blood of diabetic patient, a kind of body tissues, for the purpose of studying what metabolic changes occur in the tissues of diabetic patients. In the previous report,[1] we studied the blood ATP (adenosine triphosphate) changes of diabetic patients and healthy adults after adrenaline injection and found a remarkable difference between them. The present study was undertaken to ascertain what changes in blood ATP might be produced by oral administration of glucose.

Experimental

Method

Eight diabetic inpatients served as subjects and as a control group, eight nondiabetic inpatients and two healthy adults were selected. Blood specimens were obtained by antecubital venipuncture for the blood ATP and inorganic phosphorus determinations and by cutaneous puncture of the ear lobe for the blood sugar estimations prior to and every hour after the ingestion of 50 g. of glucose dissolved in 250 cc. of water for four hours. The blood ATP was estimated as ten minutes' hydrolyzed phosphorus by the same technique described in the first report.[1] Blood sugar determination was made by the micromethod of Fujita and Iwatake.[2] The study was performed in the overnight fasting state and in the diabetic group, this was done prior to the start of insulin treatment.

Results

Blood ATP. Speaking generally, the blood ATP level was raised by

ingestion of glucose in both the diabetic and the nondiabetic groups (Fig. 1). In the nondiabetic group, it increased definitely in seven of the ten cases, decreased in one case and remained unchanged in two other cases at the 1st hour. It decreased slightly at the 2nd hour on an average and thereafter it increased gradually. In the diabetic group, the increase in blood ATP was observed in four of the eight cases and it decreased below the fasting level in three cases at the 1st hour (Table I). On an average, it increased at the 2nd hour and kept on a high level at the 3rd and the 4th hours. The comparison of the mean maximal increase rate of both the groups revealed no statistically significant difference.

Blood inorganic phosphorus. The blood inorganic phosphorus was depressed remarkably by glucose ingestion in the nondiabetic group, but it

TABLE I

Case No.	Age Sex		Blood sugar, 10 min. P and inorg. P after glucose ingestion (mg. %) Before	1	2	3	4 hrs.
Nondiabetic group							
		Blood sugar	116	120	100	107	90
1	22 M	10 min. P	3.7	3.8	3.6	4.3	4.1
		inorg. P	5.3	4.6	4.5	4.2	4.3
		Blood sugar	102	80	85	101	121
2	32 M	10 min. P	5.1	5.5	5.4	5.4	5.9
		inorg. P	3.1	2.5	2.5	2.6	2.1
		Blood sugar	116	191	112	104	112
3	38 F	10 min.	2.8	2.8	3.0	3.1	3.2
		inorg. P	4.3	4.3	4.1	4.3	4.2
		Blood sugar	102	168	132	69	92
4	37 F	10 min. P	3.8	3.8	3.8	4.0	3.8
		inorg. P	4.2	3.7	3.6	3.4	3.8
		Blood sugar	114	193	99	71	85
5	20 M	10 min. P	3.6	3.8	3.5	3.8	3.8
		inorg. P	2.7	2.4	2.1	2.5	2.9
		Blood sugar	114	163	125	92	90
6	29 M	10 min. P	3.8	3.5	3.6	3.4	3.8
		inorg. P	4.7	4.5	4.4	4.7	4.6
		Blood sugar	111	170	92	81	97
7	36 F	10 min. P	2.8	2.9	3.0	3.1	2.9
		inorg. P	4.3	3.7	3.3	3.5	3.8
		Blood sugar	133	174	172	147	125
8	54 M	10 min. P	3.8	4.4	4.2	4.2	4.7
		inorg. P	3.8	3.6	3.5	3.4	3.3
		Blood sugar	112	130	97	90	102
9	35 F	10 min. P	4.5	5.0	4.9	4.9	4.7
		inorg. P	2.9	2.9	2.8	3.3	3.3
		Blood sugar	104	174	161	102	94
10	15 F	10 min. P	4.3	4.7	4.7	4.3	5.3
		inorg. P	5.3	4.3	3.7	3.7	4.5

Case No.	Age Sex		Blood sugar, 10 min. P and inorg. P after glucose ingestion (mg. %) Before	1	2	3	4 hrs.
Diabetic group							
		Blood sugar	284	458	358	326	294
11	48 M	10 min. P	4.0	4.2	4.3	4.6	4.1
		inorg. P	4.5	4.5	4.2	4.2	4.2
		Blood sugar	264	440	382	330	306
12	23 F	10 min. P	4.3	4.2	4.3	4.6	4.5
		inorg. P	3.7	3.8	3.7	3.8	3.8
		Blood sugar	266	388	332	287	259
13	27 M	10 min. P	4.2	4.6	4.2	4.2	4.3
		inorg. P	2.8	3.2	3.3	3.4	3.7
		Blood sugar	205	376	324	255	226
14	25 F	10 min. P	2.9	2.9	3.2	—	3.0
		inorg. P	3.3	3.3	3.3	—	3.2
		Blood sugar	174	317	268	233	182
15	39 M	10 min. P	5.0	4.7	5.0	5.6	5.3
		inorg. P	4.1	4.1	3.9	4.1	3.8
		Blood sugar	153	311	222	151	112
16	41 M	10 min. P	3.7	4.2	4.5	4.2	3.9
		inorg. P	4.0	3.9	3.1	3.1	3.0
		Blood sugar	121	217	214	161	125
17	63 M	10 min. P	4.3	4.4	4.1	4.2	4.6
		inorg. P	3.2	3.0	3.2	2.8	2.8
		Blood sugar	194	378	280	246	194
18	45 F	10 min. P	3.0	2.6	3.5	3.0	3.5
		inorg. P	4.5	4.5	3.9	4.0	3.6

decreased only slightly or not at all in the diabetic group (Fig. 2). In the healthy group, it fell abruptly to reach the minimal level at the 2nd hour in five cases and at the 3rd hour in three cases, and remained depressed until the 4th hour. In the diabetic group, on the contrary, it decreased relatively more gradually and reached the minimal level at the 4th hour in three of the six cases and interestingly the depletion in blood inorganic phosphorus was not observed at all in two of the cases. A statistically significant difference was obtained between the average maximal decrease rate of inorganic phosphorus in the diabetic group and in the nondiabetic group.

Discussion

The blood ATP represents ATP contained in blood cells, as reviewed briefly in the previous paper,[1] and is produced chiefly by anaerobic oxidation of glucose in erythrocytes because of their greater utilization of glucose compared with that in leucocytes as a whole.[3)-5)] The metabolic pathway of glycolysis in erythrocytes, however, is not clear yet. The presence of a large amount of 2,3-diphosphoglycerate in erythrocytes has been confirmed by Guest and Rapoport,[6),7)] but to what metabolic pathway it belongs

Fig. 1

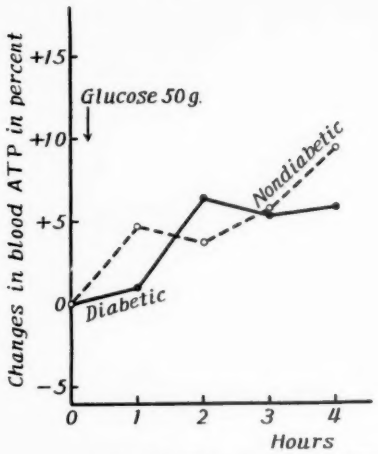


Fig. 2

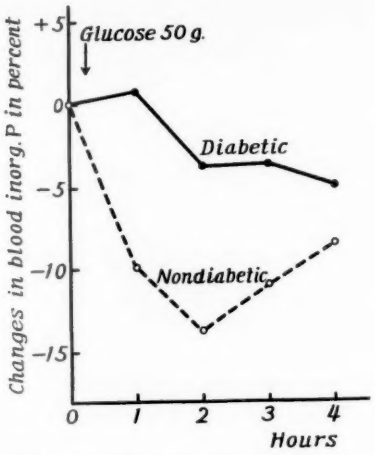


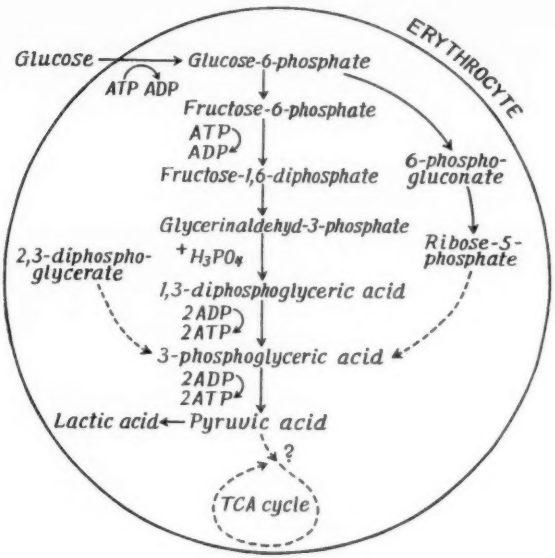
Fig. 1. Changes of blood ATP caused by glucose ingestion in percent. The solid line represents the average blood ATP level of diabetic group and broken line represents that of nondiabetic group.

Fig. 2. Changes of blood inorganic phosphorus caused by glucose ingestion in percent.

remains unsolved.[8),9)] If ATP is produced during the Embden-Meyerhof's anaerobic oxidation of glucose, two moles of ATP are formed accordingly by oxidation of one mole of glucose to lactate (Fig. 3).

The present study showed that glucose ingestion produced an increase in blood ATP in both the nondiabetic and the diabetic subjects. The rise in blood ATP after glucose ingestion was observed in the *in vivo* experiments of Kvamme[10)] and Hofmann-Credner,[11)] although such an effect was not confirmed in *in vitro* experiments.[11),12)] The mechanism of this increase in ATP formation may be presumed to consist in that the elevation of glucose content in plasma accelerates the penetration of glucose into cells and glucose is oxidized anaerobically to pyruvic acid or lactic acid. However, the reason why the increase in ATP after glucose ingestion does occur *in vivo* and not *in vitro* is hard to explain. The existence of latent disturbance of ATP formation in erythrocytes of diabetic patients was observed in our previous investigations.[1),13),14)] The present study showed that the increase in blood ATP produced by glucose ingestion is equal in diabetic as well as nondiabetic group. These two experimental results seem to be contradictory. However, in our study on arteriovenous glucose difference in glucose tolerance test, we obtained the unexpected and incomprehensible result that there was no difference in delta glucose be-

Fig. 3



tween diabetic and nondiabetic subjects in spite of the presence of the remarkable difference in arterial blood sugar level between them and we concluded from these results that the utilized amount of glucose in peripheral tissues is equal both in diabetics and nondiabetics, but the diabetic condition requires higher glycemia to utilize the normal amount of glucose; in other words, the hyperglycemia is an adaptation phenomenon of body for the diminished utilization of glucose in peripheral tissues.[15] The present result may be explained by the same way.

It is well known that the serum inorganic phosphorus falls after sugar administration, both oral and intravenous, and this depletion is relatively slight or absent in diabetic patients. The present result dealing with whole blood agreed with that obtained in serum. In this study, the rise in blood inorganic phosphorus after glucose ingestion was observed in two younger cases of the diabetic group. Such an anomalous rise in serum inorganic phosphorus was observed already by Schiller and Tyan.[16] Seino of our Clinic observed a case of acromegaly patient with diabetes who had this reverse reaction to glucose ingestion,[17] and he thought that this phenomenon is correlated closely to somatotropic hormone. Our two cases had no signs suggesting the hyperfunction of the anterior pituitary lobe.

SUMMARY

The fluctuations of blood ATP (adenosine triphosphate) and inorganic phosphorus after glucose ingestion were studied in eight diabetic and ten nondiabetic subjects. The ingestion of glucose produced an increase in blood ATP both in the diabetic and the nondiabetic groups and no statistically significant difference was obtained between these two groups. This finding is apparently contradictory to our previous findings in which a latent disturbance of blood ATP formation was confirmed in diabetic patients. The reason of this discrepancy was discussed.

The blood inorganic phosphorus was depressed by oral administration of glucose and this was much less marked in the diabetic group. Two younger diabetic cases showed a reverse reaction of blood inorganic phosphorus to the glucose ingestion.

References

1) Goto & Ito, Tohoku J. Exper. Med., 1957, **67**, 65.
2) Fujita & Iwatake, Biochem. Z., 1931, **242**, 43.
3) Bartlett & Marlow, J. Appl. Physiol., 1953, **6**, 335.
4) Guest, Mackler, Graubarth & Ammentorp, Amer. J. Physiol., 1953, **172**, 295.
5) Bird, J. Biol. Chem., 1947, **169**, 493.
6) Guest & Rapoport, Physiol. Rev., 1941, **21**, 40.
7) Guest & Rapport, J. Biol. Chem., 1939, **131**, 695.
8) Gabrio, Finch & Huennekens, Blood, 1956, **11**, 103.
9) Granick, *ibid.*, 1949, **4**, 404.
10) Kvamme, Scandinav. J. Clin. & Lab. Invest., 1951, **3**, 140.
11) Hofmann-Credner, Arch. intern. Pharmacodyn., 1956, **106**, 37.
12) Mueller & Hastings, J. Biol. Chem., 1951, **189**, 881.
13) Ito, Tohoku Igaku Z., (Jap.), 1957, **56**, 592.
14) Goto & Numazawa, Tohoku J. Exper. Med., 1958, **68**, 57.
15) Goto, Ito & Seino, unpublished.
16) Schiller & Tyan, J. Clin. Endocrinol., 1954, **14**, 828.
17) Seino, Tohoku Igaku Z. (Jap.), 1956, **54**, 205.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Fluctuations of Blood ATP, Inorganic Phosphorus and Cholesterol after Ingestion of Butter in Diabetic Patients

By

Yoshio Goto and **Yoshihiro Numazawa**
(後藤 由夫) (沼沢 吉弘)

From the Medical Clinic of Prof. T. Kurokawa, Tohoku University School of Medicine, Sendai

(Received for publication, March 1, 1958)

The metabolic disturbances in diabetes mellitus have been investigated mainly from the angle of carbohydrate metabolism and fewer studies have been attempted to elucidate the disturbances in fat and protein metabolism, which are apt to be regarded as rather side phenomena in the metabolic derangement of diabetes mellitus. It is important, however, to clarify the entity of the disturbances in fat and protein metabolism in diabetic patients, not limiting to those of experimental diabetic animals, inasmuch as an accurate knowledge is necessary for an apposite treatment of this disease.

The present study was carried out to see how the blood ATP (adenosine triphosphate), inorganic phosphorus and cholesterol contents change after the ingestion of butter in diabetic patients in comparison with healthy adults.

Experimental

Method

Five moderately severe but nonketotic diabetic inpatients and five healthy adults served as test subjects. After an overnight fast, the initial blood specimen was obtained by antecubital venipuncture without stasis for the determinations of blood inorganic phosphorus, ATP and cholesterol, then 25 g. of butter was administered orally. After the butter ingestion, the blood specimens were collected every hour for five hours. The blood ATP was estimated as ten minutes' hydrolyzed phosphorus as described in the first report.[1] The total serum cholesterol was determined by Kaye's method.[2] The test subjects were in rest during this study. This study was performed before the start of insulin treatment in diabetic patients.

Results

The results were as summarized in Table I and illustrated in Figs. 1 and 2.

TABLE I

Case No.	Age Sex		Blood 10 min. P, inorg. P and serum cholesteral after butter ingestion (mg.%) Before	1	2	3	4	5 hrs.
Healthy group								
		Blood 10 min. P	4.4	4.6	4.7	4.8	4.7	4.5
1	27 M	Blood inorg. P	3.5	3.2	3.0	3.0	3.2	3.4
		Serum cholesterol	165	165	160	165	160	172
		Blood 10 min. P	3.6	3.8	3.8	4.0	3.6	3.6
2	23 M	Blood inorg. P	4.1	4.0	3.6	3.7	3.8	3.9
		Serum cholesterol	194	199	199	193	194	205
		Blood 10 min. P	3.5	4.0	4.2	4.2	3.8	3.6
3	23 M	Blood inorg. P	3.5	3.3	3.1	3.0	3.1	3.2
		Serum cholesterol	129	126	115	115	122	122
		Blood 10 min. P	4.9	5.3	5.3	5.3	5.4	5.3
4	22 M	Blood inorg. P	5.3	4.7	4.5	4.4	4.4	4.4
		Serum cholesterol	136	132	136	132	140	144
		Blood 10 min. P	4.9	5.2	5.5	5.4	4.9	5.0
5	31 M	Blood inorg. P	3.6	3.1	2.9	3.0	3.6	3.6
		Serum cholesterol	269	269	265	259	253	259
Diabetic group								
		Blood 10 min. P	5.3	5.3	5.4	5.4	5.4	5.5
6	52 M	Blood inorg. P	4.0	3.8	3.6	3.5	3.4	3.7
		Serum cholesterol	183	180	187	—	183	193
		Blood 10 min. P	4.1	3.7	4.2	4.1	4.0	4.0
7	46 M	Blood inorg. P	4.6	4.6	4.2	4.1	4.2	4.4
		Serum chlesterol	253	256	253	256	262	253
		Blood 10 min. P	4.7	4.5	4.5	4.7	4.8	4.7
8	29 M	Blood inorg. P	4.4	4.4	4.5	4.2	4.1	4.2
		Serum cholesterol	130	114	144	124	109	126
		Blood 10 min. P	4.7	4.8	4.9	5.2	5.3	5.3
9	58 M	Blood inorg. P	2.8	3.2	2.9	2.6	2.8	2.8
		Serum cholesterol	202	199	199	202	199	191
		Blood 10 min. P	5.2	5.7	5.7	5.4	5.2	5.3
10	58 M	Blood inorg. P	4.0	3.3	3.1	3.3	3.5	3.6
		Serum cholesterol	109	109	112	111	112	109

Blood ATP. Blood ATP increased after the ingestion of butter both in healthy subjects and diabetic patients, but the increase was more prominent in the healthy group. In the healthy group, the blood ATP increased abruptly after the ingestion of butter and reached a peak at the 2nd or 3rd hour and decreased; but it didn't recover the initial level until the 5th hour. In the diabetic group, speaking generally, it increased gradually and in two cases, it continued to rise until the 5th hour. In other two of the five cases, it decreased in the first one or two hours with a following increase phase. A statistical analysis of the mean maximal increase rate gave a significant difference between these two test groups.

Blood inorganic phosphorus. A remarkable depletion in blood inorganic phosphorus was provoked by the administration of butter in both the

groups. The blood inorganic phosphorus decreased abruptly in all cases of the healthy group, reaching a minimal value at the 2nd or the 3rd hour and increased in rebound, but did not recover the initial level until the 5th hour. In the diabetic group, on the contrary, only two of the five cases showed a decrease in blood inorganic phosphorus at the 1st hour and

Fig. 1

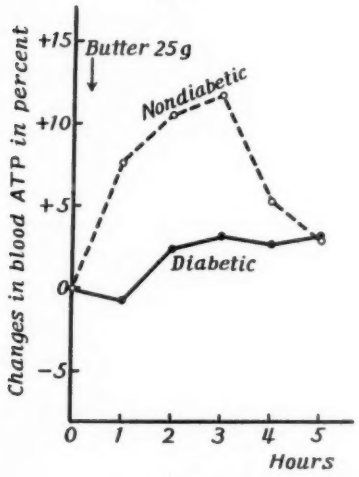


Fig. 2

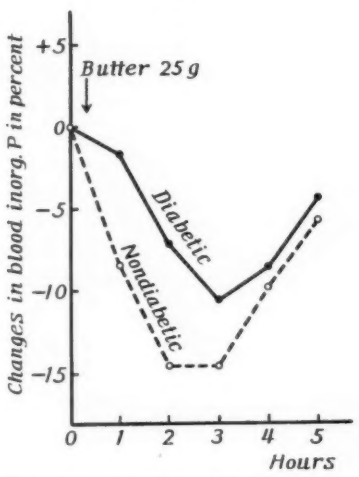


Fig. 1. Changes of blood ATP provoked by butter ingestion in percent. The solid line represents the average blood ATP level of diabetic patients and the broken line represents that of healthy adults.

Fig. 2. Changes of blood inorganic phosphorus caused by butter ingestion in percent. (Mean values).

the three other cases showed no change or increase; from the 3rd hour all the cases showed a remarkable depletion in inorganic phosphorus. A statistical analysis of the maximal decrease rate revealed that the depletion of blood inorganic phosphorus is significantly greater in the healthy than in the diabetic group.

Serum total Cholesterol. No significant fluctuation in the serum total cholesterol after butter ingestion was observed either in the diabetic or the healthy group.

Discussion

A disturbance in fat metabolism in diabetes mellitus has been pointed out by clinical observations on diabetic patients: hyperlipemia, hypercholesteremia, ketosis, etc. and a large number of investigations was attempted in

order to clarify this problem, but the experiments were performed mostly with diabetic animal tissues.

Our modern knowledge in this field may be summarized as follows.

1) The conversion of glucose to fat is impaired in diabetic rats[3)–5)] and furthermore the formation of fat from fructose,[6)] pyruvate,[7)] lactate and acetate,[8)] too (*in vitro* experiments). Chaikoff and his co-workrs concluded from their numerous brilliant works that the disturbance exist in the step of synthesis of fatty acid from acetyl CoA. These disturbances are abolished by insulin.

2) The phospholipid synthesis in liver and plasma is increased in depancreatized dog (*in vitro* experiment).[9)]

3) The conversion of acetate to glucose and glycogen is accelerated in diabetic rat liver and muscles (*in vitro*[10),11)] and *in vivo*[12),13)] experiments).

4) The incorporation of acetate carbon into cholesterol is increased in diabetic rat liver (*in vitro* experiment).[15)]

5) The production of ketone bodies increases in diabetes.

The present experimental results showed that the blood inorganic phosphorus decreased definitely after butter ingestion. Fat, decomposed into fatty acid and glycerin, are absorbed from the intestine on being phosphorylated[15)] and transported to liver or other organs via the thoracic duct in the forms of phospholipid, neutral fat, etc. Lipid and other decomposates of fat are phosphorylated in the liver[16)] and other tiessues. The depletion in blood inorganic phosphorus seems, therefore, to be a reflection of the phosphorylation of fat in body tissues and the less marked depletion in the diabetic group in comparison with the healthy group may indicate the presece of disturbance in phosphorylation of fat in diabetic patients. In what step of metabolic pathway the disturbances occur, however, is presently unclear. Interestingly, the rates of decrease in blood inorganic phosphorus are almost equal in both the conditions after ingestion of 25 g. of butter and that of 50 g. of glucose reported in other paper in nondiabetic subjects.[17)]

Concerning the mechanism of increase in blood ATP after butter ingestion, one of the possible explanations may be that ATP is produced by the anaerobic oxidation of phosphoglycerol or phosphoglyceric acid in erythrocytes, both of which are formed by phosphorylation of the glycerol portion of the fat molecule and are the intermediate metabolites of carbohydrate metabolism. The other possibility of increase in blood ATP may be due to that the amount of aerobic oxidation in leucocytes is increased by fat ingestion and as its result, high energy bound phosphates are produced in final common pathway. (Our knowledge whether mammalian erythrocytes have an aerobic oxidation system is yet far from complete. Some workers postulate the presence of the Warburg-Dickens' oxidation

pathway.[18] However, the decisive proof for the existence of tricarboxylic acid cycle in mature erythrocytes has not been obtained until today, although fragmentary experiments were attempted by Spiecer and his coworkers[19] and Nossal,[20] and the acetylation of *p*-aminobenzoic acid in erythrocytes was demonstrated by Blondheim.[21] On the other hand, it is confirmed already that the mammalian leucocytes have an active aerobic oxidation system.[22]) The fact obtained in our study that the blood ATP increased less markedly in diabetic group may indicate the decrease in phosphorylation of glycerol or the presence of a block in anaerobic oxidation of phosphoglycerol in this group and the presence of impaired aerobic oxidation of fat in diabetic leucocytes. Although there might be many probable explanations for our experimental result, speaking flatly, presently we cannot give a convincing and satisfactory explanation to this fact. We can only say that the utilization or the phosphorylation of fat is diminished in diabetic patients.

No significant fluctuation of serum cholesterol after butter ingestion was observed in this study, and this is agreeable with the results of other researchers.[23)–25)]

Summary

The changes in blood ATP (adenosine triphosphate), inorganic phosphorus and cholesterol after the ingestion of 25 g. of butter were observed in five diabetic patients and five healthy adults. The blood ATP level was raised markedly in the healthy group and it increased less markedly in the diabetic group. The depletion in blood inorganic phosphorus was provoked by butter ingestion and this was more prominent in the healthy group in comparison with the diabetic group. These facts indicate the disturbance in phosphorylation and utilization of fat in diabetic patients. The serum cholesterol did not change even after butter ingestion.

References

1) Goto & Ito, Tohoku J. Exper. Med., 1957, **67**, 65.
2) Kaye, J. Lab. & Clin Med., 1939–40, **25**, 996.
3) Stetten & Boxer, J. Biol. Chem., 1944, **156**, 271.
4) Chernick & Chaikoff, *ibid.*, 1950, **186**, 535.
5) Chernick, Chaikoff, Masoro & Isaeff, *ibid.*, 1950, **186**, 527.
6) Chernick & Chaikoff, *ibid.*, 1951, **188**, 389.
7) Osborn, Chaikoff & Felts, *ibid.*, 1951, **193**, 549.
8) Felts, Chaikoff & Osborn, *ibid.*, 1951, **191**, 683.
9) Zilversmit & Diluzio, *ibid.*, 1952, **194**, 673.
10) Strisower, Kohler & Chaikoff, *ibid.*, 1952, **198**, 115.
11) Parnes & Wertheimer, Biochem. J., 1950, **46**, 517.
12) Lifson, Lorber, Sakami & Wood, J. Biol. Chem., 1948, **176**, 1263.

13) Parnes & Wertheimer, Biochem. J., 1950, **46**, 520.
14) Hotta & Chaikoff, J. Biol. Chem., 1952, **198**, 895.
15) Verzar & Laszt, Biochem. Z., 1934, **270**, 35.
16) Cavanagh & Raper, Biochem. J., 1939, **33**, 17.
17) Goto, Ito & Ujiie, Tohoku J. Exper. Med., 1958, **68**, 51.
18) Cabrio, Finch & Huennekens, Blood, 1956, **11**, 103.
19) Spicer, Hanna & Clark, J. Biol. Chem., 1949, **177**, 217.
20) Nassal, Aust. J. Exper. Biol. & Med. Sci., 1948, **26**, 123.
21) Blondheim, Arch. Biochem. & Biophys., 1955, **55**, 365.
22) Keibl & Spitzy, Arch. exper. Path. Pharm., 1951, **213**, 162.
23) Barreda, Klin. Wschr., 1934, **13**, 290.
24) Blotner, Arch. Int. Med., 1935, **55**, 121.
25) Oppenheim & Bruger, Amer. J. Med. Sci., 1943, **205**, 77.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Chemical Nature of Toxohormone (Nakahara)

Third Report

KIK-inactive Toxohormones from Urines, Cancerous and Normal*

By

Hajime Masamune, Sen-itiroh Hakomori, Hirosi Kaketa,
(正宗 一) (箱守 仙一郎) (掛田 博)
Hyogo Sinohara and **Sin-iti Abe**
(篠原 兵庫) (阿部 辰一)

From the Medico-chemical Institute, Tohoku University, Sendai

(Received for publication, March 5, 1958)

Kaketa fraction[1] of cancerous or normal human urine was subdivided at first by dissolving in slightly alkaline water with following acidification to pH 4.5 with acetic acid. The precipitate occurring was freed from an urinary poison (Cf. a forthcoming article) by treatment with Dowex 1 and electrodialysis. The deposit that appeared on electrodialysis was chromatographed with a Dowex 1 column and a borax-HCl buffer of pH 8.4 to isolate the main component in an electrophoretically homogeneous state.

The product from cancerous urine and that from normal urine were both found to be toxohormones, although the minimum active dosis was 1 mg. in the former but 5 mg. in the latter. They lack KIK- and group-potency. Regarding their physical and chemical properties, see the experimental part.

Experimental

Preparation Procedure of the Urinary Toxohormones without KIK-potency

Kaketa fraction was prepared from mixed cancerous urines of patients with original tumour seat in stomach, liver, lung or mamma and from normal urine following that author[1]. Fractionation of those materials will be detailed on an example started with the cancerous.

5 g. of the material was stirred in water, adding 10% NaOH to pH 9.0 from time to time, and centrifuged. The centrifugate was washed with water adjusted to the same pH. The supernatant and washings were united and acidified to pH 4.5 with dilute acetic acid, and the deposit was centri-

* 213th report of Masamune and co-workers' "Biochemical Studies on Carbohydrates".

fuged off, washed with ethanol and ether and dried. 920 mg. of a yellowish brown powder was given—*Fr. a.*

It was combined with 580 mg. of a corresponding fraction after another run and taken up in 100 cc. of 0.01 *N* NaOH. The brown transparent solution of pH 10.0 was passed through a 3.6×30 cm. column of Dowex 1 at a flow rate of 30 drops per minute and washed with 400 cc. of water. The yellowish effluent (500 cc.) was electrodialyzed in a Pauli apparatus (100 volt) for 36 hours against changes of water. A precipitate occurred. It was centrifuged off, washed with ethanol and ether and dried. A grayish powder given weighed 440 mg.—*Fr. b.*

50 mg. of *Fr. b* was column-chromatographed according to Masamune *et al.*[2] as an orientating experiment. Namely, it was dissolved in 1 cc. of a borax-HCl buffer of pH 8.4 (0.05 *M* borax:0.1 *N* HCl=6:4 by volume), applied on a 0.9×20 cm. column of Dowex 1 (mesh 50–200) equilibrated with the above buffer and eluted with the same buffer at a flow rate of 4 drops per minute. The effluent was collected in fractions of 15 drops. Analytical figures of the fractions by the quantitative Molisch and biuret reactions, plotted against tube numbers, showed two peaks, the first high between tube 5 and 9 and the second very low between tube 11 and 14 (Fig. 1).

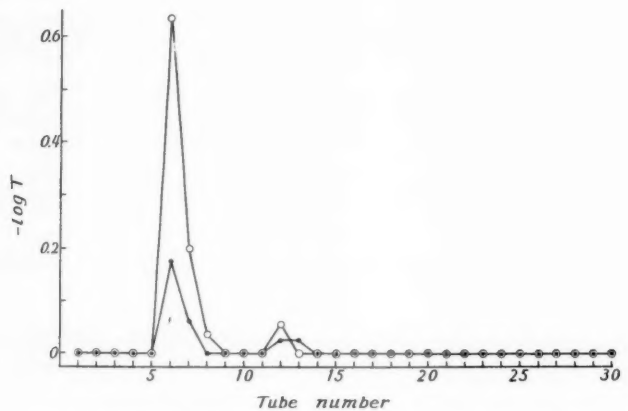


Fig. 1. Chromatogram of 50 mg. of *Fr. b* developed by the aid of a column of Dowex 1 buffered with borax-HCl buffer of pH 8.4 and the same buffer. ○-○ Molisch reaction. ●-● Biuret reaction.

In order to separate the main component, the column chromatogram was developed on a larger scale, adding 200 mg. of *Fr. b* as a solution in 4 cc. of the buffer to a 2×19 cm. column of the Dowex 1 in borate form.

When 30 cc. of the effluent had passed, the succeeding 18 cc. portion was collected and electrodialyzed for 12 hours. The precipitate was centrifuged off (The supernatant was neither Molisch- nor biuret-positive), washed with ethanol and ether and dried. 110 mg. of a grayish powder was yielded.

Average yields of the cancerous and normal products amounted to 50 and 34 mg. per liter of urine respectively. The corresponding preparations after runs were united together.

Properties of the Preparations

Toxohormone activity. It was measured in the manner described in the First Report[3]. The substance from the united cancerous urines was five times as strong as that from normal urine, whose minimum active dosis is 5 mg. per mouse. See Table IA–IC.

KIK potency. The method described by Masamune and Kawasaki[4] was applied. Both the cancerous and normal substances proved KIK inactive. Even when 2 mg. per kg. body weight of the cancerous was injected intravenously into a rabbit, the maximum descrease of erythrocytes did not reach 10% (Table II).

Blood group activity. The preparations lack ABO group activity.

Electrophoresis. The substances gave single symmetrical boundaries

TABLE IA

Liver-catalase Depression *in vivo* by the Cancerous Toxohormone (Prep. I) and the Normal (Prep. II)

The average volume of O_2 evolved for each dose in each test-set is expressed in per cent of the corresponding O_2 volume in the blank control.

No. of test-set	No. of mice	Amount of substance injected (mg.)						
		Prep. I			Prep. II			
		3	1	0.5	5	3	1	0.5
1	11	64.0	72.3					
2	9	63.4	74.7					
3	9	58.4	74.5					
4	9		70.8	101.1				
5	9		76.5	81.3				
6	6	63.7						
7	9	68.3	71.0					
8	12					80.1	84.5	93.8
9	12				75.4	79.3	88.2	
10	9				75.2	86.7		
11	6				73.4			
12	6				74.6			
13	9				72.5	80.7		
Depressed catalase activity		0.64	0.73	0.91	0.74	0.82	0.86	0.94

TABLE IB

Effects *in vivo* on Kidney- and Blood-catalase of the Cancerous Toxohormone (Prep. I) and the Normal (Prep. II)

3 mg. amounts of Prep. I and 5 mg. amounts of Prep. II were injected into mice. Liver-catalase activities were determined for comparison.

No. of mouse	Prep. I			Prep. II		
	Kidney	Blood	Liver	Kidney	Blood	Liver
Oxygen evolved (cc.) in blank experiments						
201	4.5	2.1	20.0			
202	3.8	2.5	12.2			
203	4.0	1.6	11.5			
204				3.3	1.9	10.2
205				3.0	1.7	13.8
206				3·9	2.8	11.3
Average	4.1	2.4	14.6	3.4	2.1	11.8
Oxygen evolved (cc.) in main experiments						
207	2.7	2.3	8.9			
208	4.4	2.8	10.3			
209	3.4	2.6	8.6			
210				2.8	0.7	9.0
211				3.2	2.2	7.5
212				3.5	3.4	9.7
Average	3.5	2.6	9.3	3.2	2.1	8.8
(% of the control value)	(85)	(108)	(64)	(94)	(100)	(75)

TABLE IC

Effects *in vitro* on Liver-catalase of the Cancerous Toxohormone (Prep. I) and the Normal (Prep. II)

The liver homogenates were incubated with 3 mg. of Prep. I or 5 mg. of Prep. II.

No. of mouse	Liver catalase activity (oxygen evolved in cc.)					
	Prep. I			Prep. II		
	Control I (No incubation)	Control II (Incubated without the substance)	Main (Incubated with the substance)	Control I (No incubation)	Control II (Incubated without the substance)	Main (Incubated with the substance)
213	11.4	9.8	10.8			
214	13.4	11.6	10.9			
215	12.6	11.0	11.5			
216				11.8	8.9	9.2
217				9.4	7.2	6.9
218				10.5	7.7	8.5
Average	12.1	10.8	11.1 (103% of Control II value)	10.6	7.9	8.2 (104% of Control II value)

TABLE II

KIK-potencies of the Cancerous Toxohormone (Prep. I) and of the Normal (Prep. II)

Rabbit No.	Body wt.(kg.)	Sex	Material injected and dosis per kg. body weight (mg.)	Number of red cells: Before injection	Time after injection (hrs.) 1	2	3	4	5	Maximum decrease (%)
1	2.4	♂	Prep. I 2.0	711	760	736	692	**650**	689	8
2	2.9	♀	" "	701	756	721	753	**648**	701	7
3	2.2	♂	" "	689	634	729	**634**	655	652	9
4	2.2	♂	" "	630	619	659	616	**590**	603	6
5	2.1	♂	Prep. II "	688	765	792	814	720	**687**	0
6	2.1	♀	" "	423	443	**426**	490	477	480	0

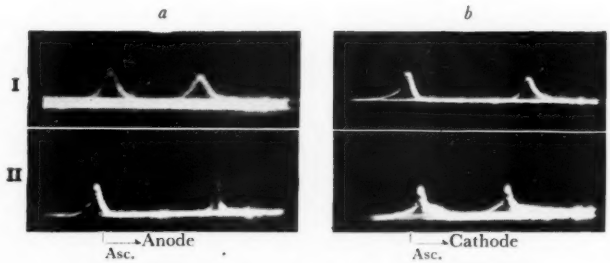


Fig. 2. Electrophoresis patterns (4/5×) of 1% solutions of the cancerous toxohormone (**I**) and the normal (**II**) in veronal-HCl buffer, pH 8.6, I=0.2 (*a*) and glycine-HCl buffer, pH 2.0, I=0.2 (*b*); current 4 mA; temperature 10°C; exposed 2 hrs. after starting current. Mobility:

$$\mathbf{I}a \quad \frac{(-0.8)\times 0.0095\times 0.18}{7200\times 0.004} = -4.7\times 10^{-5}\ \mathrm{cm^2/volt/sec.}$$

$$\mathbf{I}b \quad \frac{(+0.8)\times 0.0158\times 0.18}{7200\times 0.004} = +9.8\times 10^{-5}\ \text{"}$$

$$\mathbf{II}a \quad \frac{(-0.88)\times 0.0095\times 0.18}{7200\times 0.004} = -5.2\times 10^{-5}\ \text{"}$$

$$\mathbf{II}b \quad \frac{(+0.4)\times 0.0158\times 0.18}{7200\times 0.004} = +4.2\times 10^{-5}\ \text{"}$$

after electrophoresis in an acid and an basic buffer, as shown in Fig. 2. It is noteworthy that their mobilities in either of the buffers differ from each other.

Optical rotation. The preparations were taken up in 0.005 *N* NaOH, and the solutions of pH 9.0 were examined.

The cancerous substance:

$$[\alpha]_D^{\tau} = \frac{-0.138 \times 100}{0.5 \times 0.25} = -110°$$

The normal substance:

$$[\alpha]_D^{\tau} = \frac{-0.126 \times 100}{0.5 \times 0.25} = -100°$$

Ultraviolet absorption. 25 mg% solutions of the substances were prepared by dissolving in water by addition of NaOH. The solutions given were of pH 9.0. The spectral absorptions were examined in a photoelectric spectrophotometer (Type EPU-2, Hitachi). The cancerous substance absorbed ultraviolet light a little more strongly than the normal, the maximum being between 280–285 mμ for both of the substances (Fig. 3).

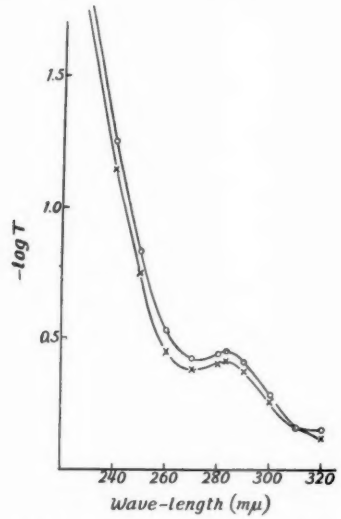


Fig. 3. Ultraviolet absorption spectra of 25 mg% solutions (pH 9.0) of the cancerous toxohormone (○—○) and the normal (×—×). Cell 1 cm. thick.

Solubility. The cancerous substance behaves similar to the normal in this respect. They dissolve in water, when alkali is added to pH 8.4, and precipitate on acidification to pH 4.4–4.6. They are insoluble in methanol, ethanol, ether, acetone and glacial acetic acid, but soluble in 90% phenol. Their solutions of pH 7.0 (The substances were dissolved in water by the aid of alkali and adjusted to pH 7.0 before examination) are precipitated not only when saturated with sodium sulfate at 35°C or with ammonium sulfate (room temperature) but also on addition of cupric chloride, lead acetate and trichloroacetic acid. They are not precipitated with barium

acetate.

Test-tube tests. The results will be tabulated.

Test	Product	
	Cancerous	Normal
Molisch	++	++
Biuret	+	+
Osaki-Turumi		
Direct*	−	−
Indirect*	−	−
Bial-Mejbaum	−	−
Sakaguchi	−	−
Test for phosphorus	−	−

* 0.5% solutions of the substances were tested.

Paper partition chromatography of the components. Sugars. 22–23 mg. of substance was hydrolyzed with 1.5 cc. of 1 N H_2SO_4 by heating in a sealed tube at 100°C for 5 hours. The hydrolysate was neutralized to pH 5.0–6.0 with the saturated baryta, the precipitate was centrifuged off and washed with changes of water at 70°C, and the supernatant and washings were evaporated together to dryness in vacuo over $CaCl_2$. The residue was dissolved in 0.04 cc. of water, and two 12×60 cm. slivers of Toyo Roshi filter paper No. 2 were spotted each with a half of the solution to chromatograph according to Masamune and Yosizawa[5]. See Fig. 4. The substances, cancerous and normal, were both found to contain galactosamine, glucosamine, galactose, mannose, glucose and L-fucose. No spots were indicated at the positions corresponding to xylose or lyxose in the *n*-butyl-acetate-acetic acid-ethanol-water chromatograms. Its presence was also negated by a method of spectrophotometric pentose analysis.[6]

Amino acids. 3 mg. substance was heated with 0.5 cc. of 6 N HCl in a sealed tube at 100°C for 24 hours, allowed to cool and repeatedly evaporated at below 40°C in a vacuum desiccator containing solid caustic soda and anhydrous calcium chloride, adding 2–3 drops of water from time to time, until the solution showed a pH value higher than 2.6. The solution thus freed from most of the hydrochloric acid was diluted with water and filtered with suction. The filtrate was evaporated in the desiccator to dryness and dissolved in 0.12 cc. of water. 0.02 cc. of the solution was applied on a 30×30 cm. square of Toyo Roshi filter paper No. 50 to develop a two-dimensional chromatogram. The solvent used for the primary irrigation was butanol-acetic acid-water (4:1:2 by volume) and that used for the secondary irrigation was phenol-0.1 M borax (8:2 by volume). The chromatogram obtained and dried was sprayed at first with 20% acetic acid, and after again drying, with 0.2% ninhydrin in moist butanol. In both the cases of the cancerous and normal products, spots were shown at

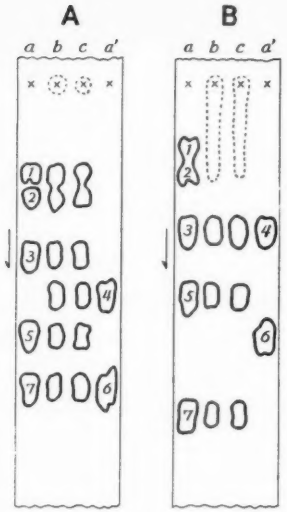


Fig. 4. Sugar paper chromatograms of the cancerous toxohormone and of the normal, developed in a descending way with solvent butanol-pyridine-water (5:3:2 by volume) (**A**) or solvent *n*-butylacetate-acetic acid-ethanol-water (3:2:1:1 by volume) (**B**) and indicated with aniline hydrogen phthalate reagent of Partridge. Temperature and duration of irrigation 25°±1°C and 22 hrs. respectively. Reference runs carried out with 0.02 cc. of solutions which were 1% with respect to individual non-amino sugars and amino sugar hydrochlorides.

a, *a'*: reference. *b*: the cancerous toxohormone. *c*: the normal toxohormone. *1* galactosamine, *2* glucosamine, *3* galactose, *4* glucose, *5* mannose, *6* xylose, *7* L-fucose.

the positions corresponding to aspartic and glutamic acids, serine, glycine, threonine, hexosamine, lysine, histidine, tyrosine, alanine, proline, valine and/or methionine, leucine and/or isoleucine, and phenylalanine. See Fig. 5.

The substances looked thus to be devoid of arginine. Nakahara and Fukuoka[7], however, said that arginine, phenylalanine and leucine were necessary and sufficient amino acids for their biosynthesis of toxohormone. Therefore, whether arginine is actually absent or not in our preparations was scrutinized as follows:—10 mg. substance was hydrolyzed with 6 *N* hydrochloric acid for 20 hours, and the hydrochloric acid was expelled as above. The dry residue was dissolved in 5 cc. of water, passed through a column of Amberlite IRC 50 (0.5×3 cm.) and washed with 50 cc. of water until the effluent became ninhydrin negative. Then the absorbed compo-

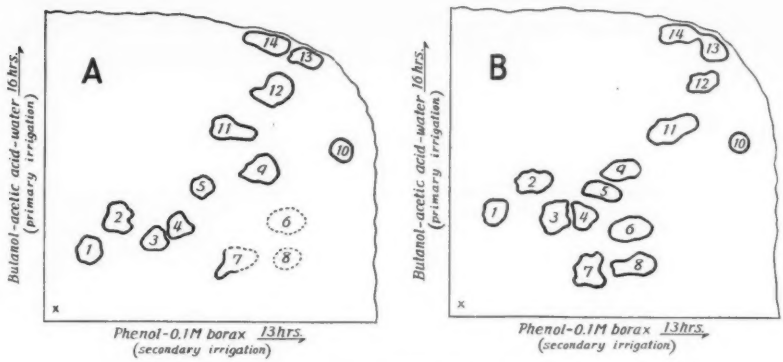


Fig. 5. Amino acid paper chromatograms of the cancerous toxohormone (**A**) and of the normal (**B**), developed with the mixtures of butanol, acetic acid and water and of phenol and 0.1 *M* borax at 25°±1°C. Indicator 0.2% ninhydrin in moist butanol.

1 aspartic acid, *2* glutamic acid, *3* serine, *4* glycine, *5* threonine, *6* hexosamine, *7* lysine, *8* histidine, *9* alanine, *10* proline, *11* tyrosine, *12* valine and/or methionine, *13* leucine and/or isoleucine, *14* phenylalanine.

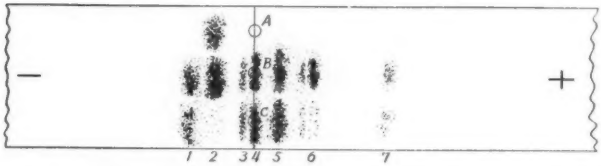


Fig. 6. Paper-electrophoresis pattern of the fractions adsorbed to Amberlite IRC 50 of the hydrolysate of the toxohormones from cancerous and normal urines. Paper spotted with the fractions was held horizontally on a special frame of Kobayashi, and after electrophoresis, sprayed with 0.2% ninhydrin in a mixture of moist butanol and glacial acetic acid (1:1).

Open circles represent starts. *A*: reference run with arginine. *B*: toxohormone from cancerous urine. *C*: toxohormone from normal urine. *1* unknown spot (brownish red), *2* arginine (purple), *3* proline (yellow), *4* lysine (purple), *5* histidine (blue), *6* and *7* unknowns (purple).

nents were eluted with 50 cc. of 1 *N* formic acid and distilled in vacuo, and a 1/5 portion of the still-residue was put on a paper 33×6 cm. and subjected to electrophoresis in 0.05 *M* borax (pH 9.6) at constant 600 V (Initial current 1.3 mA per cm. width) for 6 hours. As shown in Fig. 6, the hydrolysate of the cancerous toxohormone gave a strong spot of arginine but that of the normal toxohormone only a faint spot of the same amino acid.

Quantitative analyses. The analytical figures obtained are embodied in Table III. The cancerous and normal substances quite agree with each other in content of the sugar components excepting glucose.

TABLE III

Composition of the Cancerous Toxohormone (Prep. I) and the Normal (Prep. II)

Analysis	In per cent		In equivalents per equivalent weight	
	Prep. I	Prep. II	Prep. I	Prep. II
N*	13.5	13.8		
Hexosamine†	3.0	2.9	1.00	1.00
Galactose‡	1.6	1.9	0.52	0.63
Glucose‡	2.0	1.0	0.65	0.33
Mannose‡	1.5	1.4	0.48	0.47
L-Fucose§	0.4	0.5	0.14	0.15
Sialic acid//	3.4	4.4	0.63	0.86
Ash¶	0.0	0.6		

* Micro Kjeldahl. † Masamune and Yosizawa.[8] ‡ Masamune and Sakamoto.[9] § Masamune and Sakamoto.[10] // Werner and Odin[11] (the direct Ehrlich) ; Calcd. as $C_{11}H_{19}NO_9$. ¶ Pregl method without use of H_2SO_4.

SUMMARY

1. Toxohormones without KIK potency have been isolated in a pure state from cancerous and normal urines. The yield of the normal substance was less than that of the cancerous.

2. The cancerous toxohormone is about five times as active as the normal. They are devoid of ABO group activity.

3. The physical and chemical properties of those substances are described.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

References

1) Kaketa, Tohoku J. Exp. Med., 1958, **67**, 319.
2) Masamune, Kaketa & Abe, S., *ibid.*, 1958, **67**, 329.
3) Masamune, Tsuiki, Kamiyama, Abe, S., Haga, Kawasaki, Kaketa & Abe, Sh., *ibid.*, 1958, **67**, 309.
4) Masamune & Kawasaki, *ibid.*, 1956, **63**, 369.
5) Masamune & Yosizawa, *ibid.*, 1953, **59**, 1.
6) Masamune & Sakamoto, *ibid.*, 1956, **63**, 357.
7) Nakahara & Fukuoka, Gann, 1954, **45**, 77.

8) Masamune & Yosizawa, Tohoku J. Exp. Med., 1956, **64**, 53.
9) Masamune & Sakamoto, *ibid.*, 1956, **63**, 345.
10) Masamune & Sakamoto, *ibid.*, 1956, **63**, 363.
11) Werner & Odin, Acta soc. med. Upsal., 1952, **57**, 230.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Effects of Low Oxygen Inhalation on Values of Electric Flicker

By

Kiichiro Suzuki and **Yoshio Toratani**
(鈴木 亀一郎) (虎渓 良男)

From the Physiological Laboratory of Prof. K. Motokawa, Tohoku University, Sendai

(Received for publication, March 6, 1958)

Introduction

Motokawa and Suzuki[1] showed that a flicker sensation caused by electric stimulation of the eye can be used as a very convenient indicator for the measurement of fatigue. Motokawa and Iwama[2] reported that a remarkable increase of electric flicker values (EFV) was caused by oxygen lack which was produced by low atmospheric pressure.

In the present experiment this problem was investigated in another way, measuring EFV in response to inhalation of a mixture of nitrogen and oxygen (10% O_2). In addition, the value of arterial oxygen saturation was measured by means of an ear oximeter.

Experimental

Method

The subjects in the present experiment were 7 healthy males. For 30 minutes before each experiment, the subjects were laid down quietly on the bed, breathing the room air through a mouthpiece. The measurements of EFV and arterial oxygen saturation were carried out for 10 minutes after the rest to obtain control values. The subjects were made anoxemic by inhalation of 10% O_2 immediately after the measurement of the control values. Similar measurements were made continuously during and after inhalation of 10% O_2.

The apparatus for electric flicker used in the present experiment was the same as was described in Motokawa's report[3] in detail. The stimulating current was increased and decreased automatically, to determine the threshold for appearance of flicker and that for its disappearance. The difference of both thresholds S_1–S_2 was denoted by EFV or ΔS. The EFV was expressed in terms of μA.

The apparatus used for the measurements of arterial oxygen saturation was the ear oximeter of Wood type.[4]

Results

In 3 subjects, the period of low oxygen inhalation was 3 minutes. The results obtained from these subjects are plotted in Fig. 1 (I, II and III). As can be seen from this figure, EFVs for each subject increased promptly

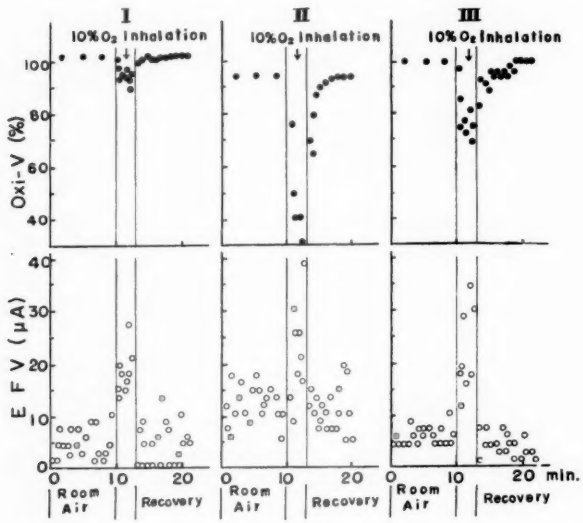


Fig. 1. Changes of electric flicker value (EFV) and ear oximeter value (Oxi-V) following inhalation of 10% O_2. I, II and III represent data of different subjects.

immediately after inhalation of 10% O_2, while oximeter values (Oxi-V) decreased. Recovery was very prompt with regard to the EFV, but slower with regard to the Oxi-V; it took 2–5 minutes for the Oxi-V to reach the initial value. It is without saying that the difference between EFVs before and during the 10% O_2 inhalation is statistically significant.

In other two subjects (Nos. IV and V), 10% O_2 inhalation of 3 minutes was made twice with a certain interval; the interval was 5 minutes for subject No. IV and 10 minutes for subject No. V. The results are illustrated in Fig. 2 (IV and V). In the first administration of 10% O_2, the amount of increase in EFV and that of decrease in Oxi-V were similar to those obtained in subjects Nos. I, II, and III, but in the second administration of 10% O_2 the change in EFV was much smaller than in the first.

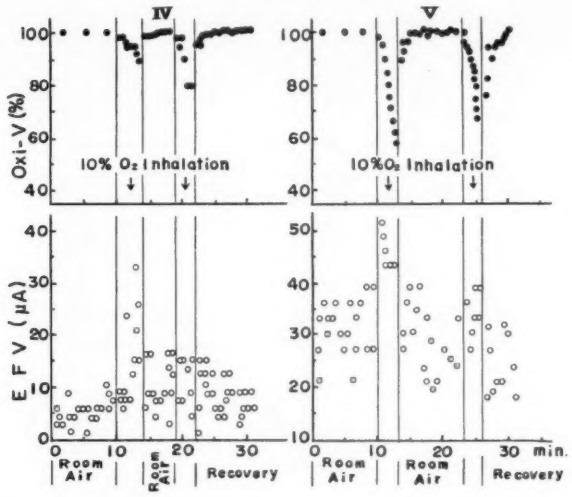


Fig. 2. Changes of EFV and Oxi-V following inhalation of 10% O_2.

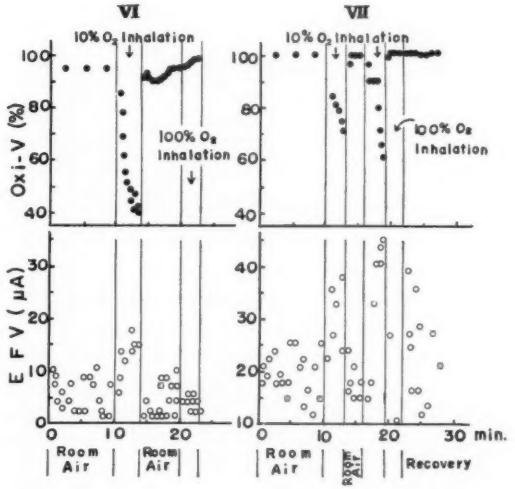


Fig. 3. Effect of pure oxygen on changes in EFV and Oxi-V caused by 10% O_2 inhalation.

In the following experiment it was tested whether or not recovery was facilitated by inhalation of 100% O_2. In subject, VI, 6 minutes after the end of inhalation of 10% O_2, the 100% O_2 inhalation was made. No

significant effect could be found on EFV and Oxi-V during the 100% O_2 inhalation. In subject No. VII, immediately after the end of the second inhalation of 10% O_2, 100% O_2 was administrated for 2.5 minutes. In this case the effect was conspicuous; both EFV and Oxi-V recovered promptly to the control values, as is shown in Fig. 3 VII. However, the EFV thus restored to the initial value increased again when the 100% O_2 inhalation was stopped, and then recovered gradually.

The changes in Oxi-V caused by 10% O_2 inhalation show remarkable individual variations, as can be seen from Figs. 1–3. The same can be said about its initial values. Then the question arose as to whether there might be some correlation between the initial value and the change caused by 10% O_2 inhalation. In Fig. 4A the changes found in 7 subjects was

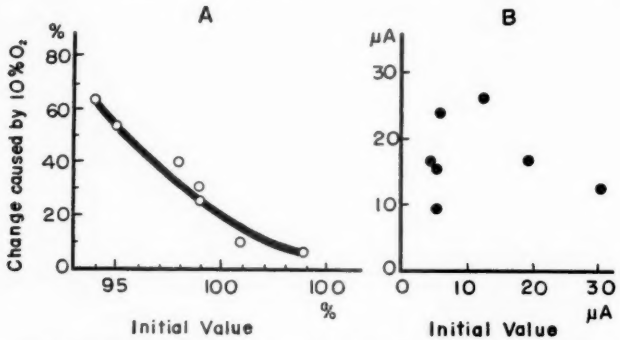


Fig. 4. Relation between initial values and corresponding changes caused by 10% O_2 inhalation. A refers to Oxi-V, and B to EFV. Difference between initial value and value at end of 3 minutes' inhalation of 10% O_2 was taken as change.

plotted as ordinates against the corresponding initial values as abscissae. As can be seen in this figure, the larger the initial values, the smaller the changes caused by 10% O_2 inhalation. No definite correlation could, however, be obtained between the initial EFVs and the corresponding changes caused by 10% O_2 inhalation, as can be seen in Fig. 4B.

In the following experiment similar measurements were made before, during and after exercise, a steptest consisting of going up and down 2 stairs, 25 cm. high each, at a constant rate (15 times per minute). The steptest continued for 20 minutes, and in the latter half of this period subjects were ordered to inhale 100% O_2. The results obtained are shown in Fig. 5. As can be seen in this figure, the EFV increased and the Oxi-V decreased almost in the same way by the exercise. On application of 100% O_2 the Oxi-V increased promptly, but its effect was only tem-

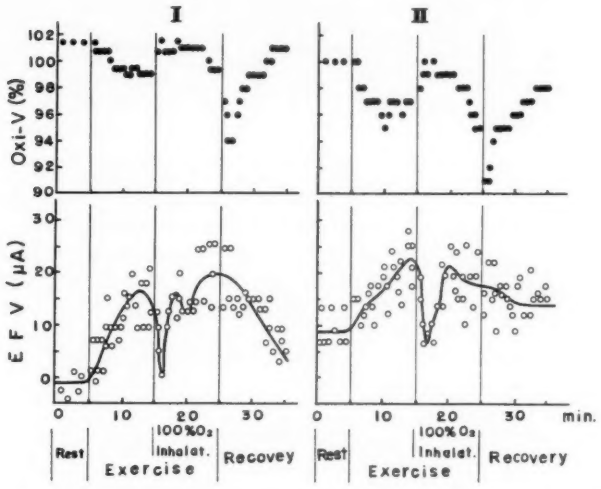


Fig. 5. Effect of exercise, a steptest, upon EFV and Oxi-V and its modification by inhalation of pure oxygen.

porary; it decreased again in spite of the continuous inhalation of 100% O_2. The effect of 100% O_2 on the EFV was also temporary, and decayed earlier than the effect on the Oxi-V. After the exercise recovery went on slowly and similarly with both EFVs and Oxi-V.

Discussion

Sasamoto[5] reported that it took about 3–5 minutes to reach a steady low Oxi-V after onset of low oxygen inhalation (4%–16%). The same has been observed in the present experiment. Changes of EFV occurred more promptly than the corresponding changes in Oxi-V. In other respects both changes occurred in parallel. Therefore it may be supposed that the change in EFV is a secondary effect of oxygen deficiency caused either by inhalation of low oxygen or indirectly by severe exercise, but the EFV can not serve as a direct indicater of anoxia, because it must depend not only on anoxia, but also on other factors. The same can be said also from the difference observed above in the time-courses of the EFV and Oxi-V.

It is to be noted that the administration of 100% O_2 during exercise showed only a temporary effect. The effect was very marked at the onset of inhalation of pure oxygen, especially on the EFV, but subsided soon for unknown reasons. This change can not be ascribed to the change in Oxi-V, because the former occurred much in advance to the latter.

SUMMARY

By means of Motokawa's method of electric flicker, we measured variations of electric flicker values (EFV) of 7 subjects during and after 10% O_2 inhalation. Measurements by an ear oximeter were carried out in parallel.

During 3 minutes' inhalation of 10% O_2 EFVs increased, but oximeter values (Oxi-V) decreased, the change in EFVs being more prompt. Recovery occurred with similar time-courses in both phenomena.

In severe exercise both kinds of values changed similarly to the insufficient oxygen supply caused above. Administration of pure oxygen during exercise reduced the changes only temporarily.

The authors have pleasure in acknowledging their indebtedness to Prof. Motokawa who has guided them kindly.

References

1) Motokawa, K. & Suzuki, K., Jap. Med. J., 1948, **1**, 200.
2) Motokawa, K. & Iwama, K., Tohoku J. Exp. Med., 1949, **50**, 319.
3) Motokawa, K., Umetsu, J., Kobayashi, M. & Kameyama, M., *ibid.*, 1956, **63**, 151.
4) Wood, H., J. Lab. Clin. Med., 1949, **34**, 387.
5) Sasamoto, H., Sogo Rinsho (Jap.), 1955, **4**, 19.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Molisch-positive Mucopolysaccharides of Gastric Cancers as Compared with the Corresponding Components of Gastric Mucosae

First Report

Fractionation Procedure of Gastric Cancer and Gastric Mucosa*

By

Hajime Masamune, Hiroya Kawasaki, Sin-iti Abe,
(正宗 一) (川崎裕也) (阿部辰一)
Koki Oyama and **Yoshitomo Yamaguchi**
(小山光紀) (山口善友)

From the Medico-chemical Institute, Tohoku University, Sendai and Takeda General Hospital, Aizu-Wakamatsu

(Received for publication, March 9, 1958)

Several papers[1)-4)] have been already published from this Medico-chemical Institute regarding the group mucopolysaccharides of gastric cancers and of gastric mucosae to establish how the cancerous group substances deviate in composition from the corresponding non-cancerous. However, the methods of isolating high-molecular components from animal tissues and of analyses of them have been recently amazingly improved, necessitating a reinvestigation. Fortunately, large amounts of the same materials as above have become accessible to us by favour of a grant from the Ministry of Education and friendly support of many hospitals. Therefore, a thoroughgoing chemical study has been undertaken anew to carry out on as many mucopolysaccharides as obtainable from those tissues, including those which are group active, with respect not only to their composition but also to their structure.

The present account deals with a great part of the procedure for separating the tissue components in view in a pure state.

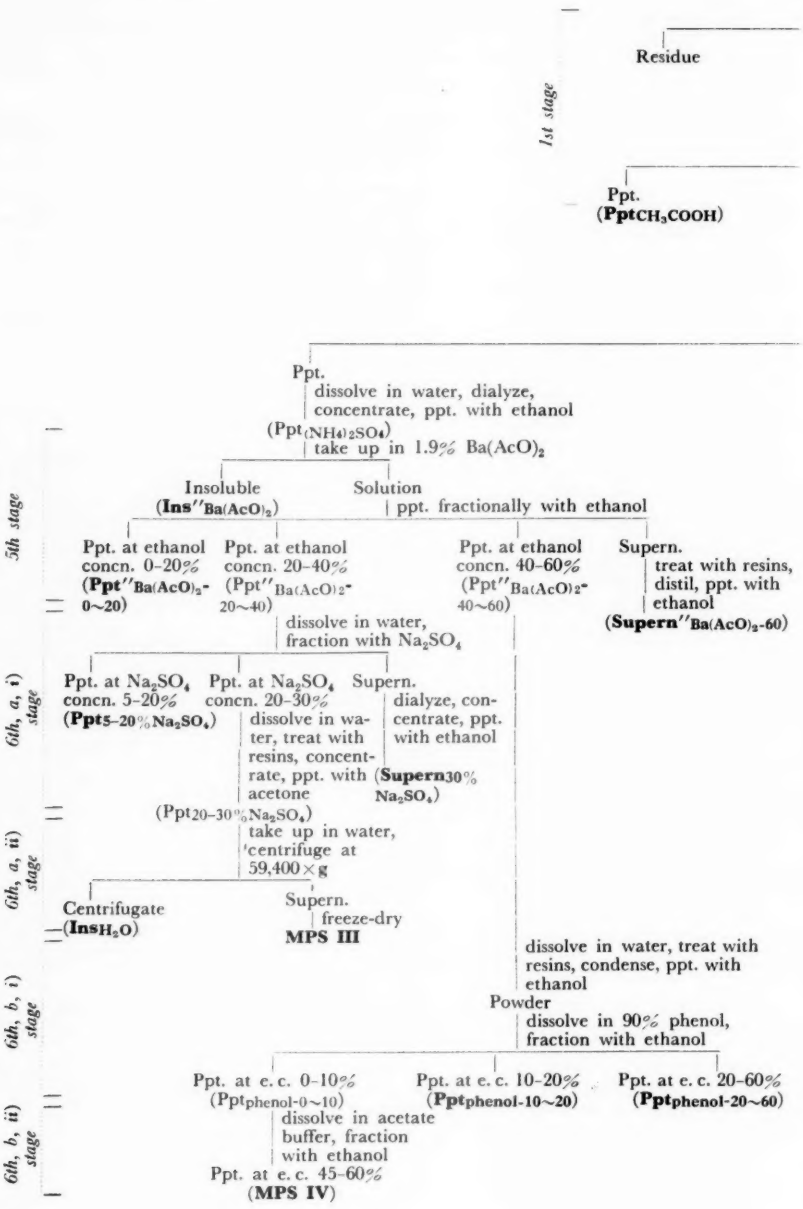
Experimental

Materials

Gastric cancers and mucosae of non-cancerous stomachs, which had been covered separately according to blood group A, B or O under

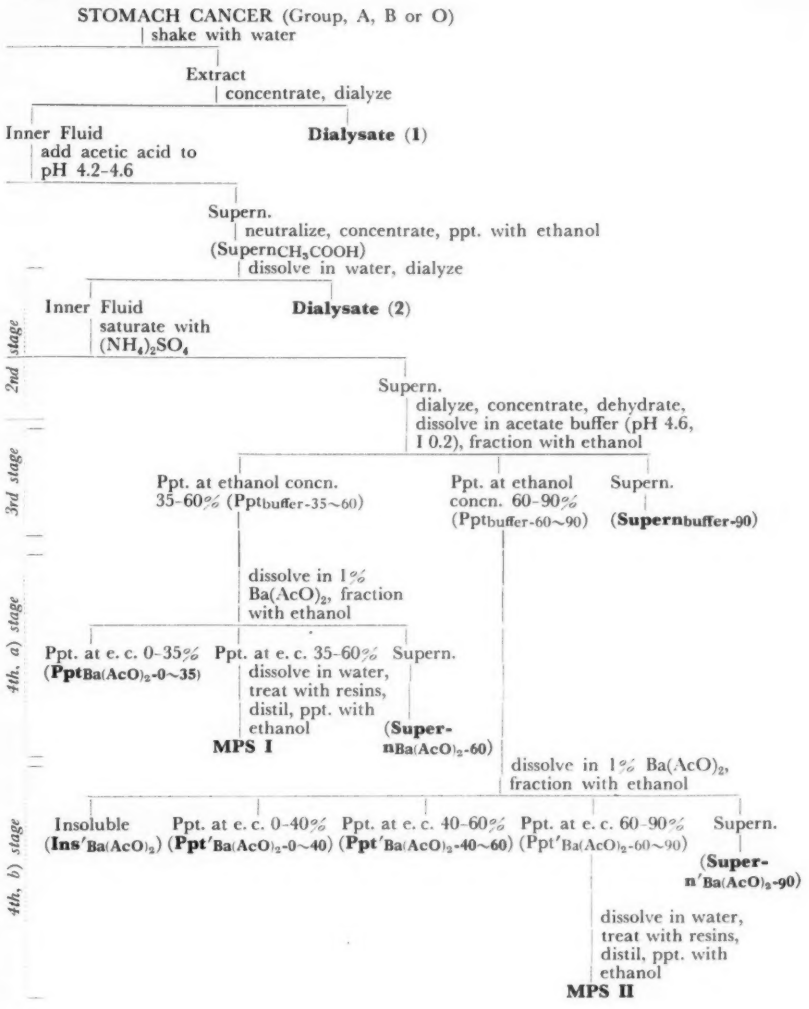
* 214th report of Masamune and co-workers' "Biochemical Studies on Carbohydrates." A part was read before the XVIth General Meeting of the Japanese Cancer Association, Osaka, October 30-31, 1957.

TABLE
Isolation of



I

MPSs I–IV


STOMACH CANCER (Group, A, B or O)
shake with water
Extract
concentrate, dialyze
Inner Fluid
add acetic acid to pH 4.2-4.6
Dialysate (1)
Supern.
neutralize, concentrate, ppt. with ethanol
(Supern$_{CH_3COOH}$)
dissolve in water, dialyze
Inner Fluid
saturate with $(NH_4)_2SO_4$
Dialysate (2)
2nd stage
Supern.
dialyze, concentrate, dehydrate, dissolve in acetate buffer (pH 4.6, I 0.2), fraction with ethanol
3rd stage
Ppt. at ethanol concn. 35-60% (Ppt$_{buffer-35\sim60}$)
Ppt. at ethanol concn. 60-90% (Ppt$_{buffer-60\sim90}$)
Supern. (Supern$_{buffer-90}$)
dissolve in 1% $Ba(AcO)_2$, fraction with ethanol
4th. a) stage
Ppt. at e. c. 0-35% (Ppt$_{Ba(AcO)_2-0\sim35}$)
Ppt. at e. c. 35-60%
dissolve in water, treat with resins, distil, ppt. with ethanol
MPS I
Supern. (Super-n$_{Ba(AcO)_2-60}$)
dissolve in 1% $Ba(AcO)_2$, fraction with ethanol
4th. b) stage
Insoluble (Ins'$_{Ba(AcO)_2}$)
Ppt. at e. c. 0-40% (Ppt'$_{Ba(AcO)_2-0\sim40}$)
Ppt. at e. c. 40-60% (Ppt'$_{Ba(AcO)_2-40\sim60}$)
Ppt. at e. c. 60-90% (Ppt'$_{Ba(AcO)_2-60\sim90}$)
Supern. (Super-n'$_{Ba(AcO)_2-90}$)
dissolve in water, treat with resins, distil, ppt. with ethanol
MPS II

4–5 volumes of ethanol for various durations from 1/2 month to 2 years, were squeezed, masticated, and after drying in a Faust apparatus, pulverized. The six different materials were further dried in a desiccator ($CaCl_2$).

Isolation Procedure of the Mucopolysaccharides

The case started with the dry group A cancer material will be described as an example. The principle is illustrated in Table I.

First stage. 180 g. of the dry material was shaken with 2 *l.* of water for 2 days at room temperature (20°C), avoiding foam formation with 5 drops of octyl alcohol. The insoluble residue was further shaken with four 1.3 *l.* portions of water in succession, each extraction taking 1 day. The first yellowish turbid extract of pH 7.0—**Extract 1**—which amounted to 1.4 *l.* was distilled in vacuo to 300 cc. and dialyzed against 1.5 *l.* of water for 12 hours. The yellowish, transparent dialysate was distilled under reduced pressure to a syrup and placed in a vacuum-desiccator ($CaCl_2$). 4.5 g. of a hygroscopic yellow powder was given—**Dialysate 1**. The inner fluid in the dialysis bag (500 cc.) was acidified to pH 4.5 with 20% acetic acid. A white flocculent precipitate that occurred was centrifuged off, washed with water adjusted to pH 4.5, ethanol and acetone in turn and dried. 1.6 g. of a gray fibrous substance was given—**Ppt$_{CH_3COOH}$**. The mother fluid (yellowish turbid) and watery washing were combined, filtered through a cellulose pad with suction, neutralized to pH 6.0 with NaOH and distilled in vacuo to a syrup. It was precipitated with 200 cc. of ethanol, washed with ethanol and acetone and dried. 6.5 g. of a yellowish powder was given—**Supern$_{CH_3COOH}$**.

The successive tissue extracts were treated similar to above with yields of the fractions following:

No. of extract	Yield (g.).		
	Dialysate 1	Ppt$_{CH_3COOH}$	Supern$_{CH_3COOH}$
1	4.51	1.55	6.46
2	2.25	1.70	1.92
3	0.73	1.10	0.84
4	0.55	0.89	0.30
5	0.30	0.85	0.28
Total	8.34	6.09	9.80

Second stage. 9.0 g. of Supern$_{CH_3COOH}$ was dissolved in 90 cc. of water and dialyzed for 24 hours against 1.5 *l.* and 1.2 *l.* portions of water in succession, and the yellow transparent dialysates were concentrated together by distilling in vacuo to dryness to give 3.5 g. of a hygroscopic yellow substance—**Dialysate 2**. The almost clear inner fluid was distilled under reduced pressure to about 200 cc., saturated with 150 g. of ammonium

sulfate and acidulated to pH 5.6 with 3% sulfuric acid. The flocculent deposit was centrifuged off after standing for 3 hours at room temperature (15°C) and reprecipitated twice from decreasing volumes of water. Then it was taken up in 120 cc. of water, dialyzed against running water for 3 days and distilled in vacuo to syrupy consistency, and 150 cc. of ethanol containing some anhydrous sodium acetate was added. The precipitate here was separated, washed with ethanol and ether and desiccated. 2.94 g. of a grayish powder with yellow hue was given—**Ppt**$_{(NH_4)_2SO_4}$. The filtrates saturated with $(NH_4)_2SO_4$ (yellowish transparent) were united, diluted with 2 volumes of water, and after 2 days' dialysis against running water, condensed to about 1/10 the volume, followed by dialysis for the same duration of time. The fluid was then distilled to a syrup, precipitated, washed and dried as when Ppt$_{(NH_4)_2SO_4}$ above was separated. 0.93 g. of a white powder was given—**Supern**$_{(NH_4)_2SO_4}$.

Third stage. 0.93 g. of Supern$_{(NH_4)_2SO_4}$ was dissolved in such a volume of 0.2 *M* acetate buffer of pH 4.6 (I 0.2) as gives a 2% solution. To the yellow, almost clear solution was added 99% ethanol drop by drop. Turbidity began to occur at the ethanol concentration of 35% (v/v) and turned flocculent at that of 60%. The adhering precipitate was centrifuged off after standing for 1 hour. It was reprecipitated from 10 cc. of the same buffer in a similar manner, washed with ethanol and ether and dried. A white powder given—**Ppt**$_{\text{buffer-35}\sim60}$—weighed 440 mg. The supernatant fluids containing 60% ethanol were combined, and after further addition of ethanol to 90%, stood for 2 hours. The centrifugate, which was not adhering at all, was washed and dried as above. 440 mg. was the white powder given here—**Ppt**$_{\text{buffer-60}\sim90}$. The centrifuged supernatant was distilled in vacuo to a syrup, dehydrated with excess ethanol, washed and dried as above. Yielded was 10 mg. of a substance—**Supern**$_{\text{buffer-90}}$.

The fractional precipitations above were processed at 0°C. Operations at 15°C entail the same result.

Fourth stage. a) 435 mg. of Ppt$_{\text{buffer-35}\sim60}$ was taken up in 50 volumes of 1% $Ba(AcO)_2$. To the faintly opalescent solution cooled on ice was added ethanol in drops. Turbidity began to increase at the ethanol concentration of 10% and changed flocculent at that of 30%. Ethanol was added additionally to 35%, the mixture was stood for 1 hour in ice-water, and the non-sticky precipitate was centrifuged off and washed with three 6 cc. portions of a solution containing as much $Ba(AcO)_2$ and ethanol as the mother fluid did—**Ppt**$_{Ba(AcO)_2\text{-}0\sim35}$. The centrifuged supernatant and washing were combined, and the ethanol concentration was raised to 60% similar to above, whereby a sticky precipitate occurred. After standing for 2 hours under continuous cooling, the deposit was centrifuged off,

washed with ethanol and dried. It was next dissolved in 30 cc. of water, and the clear solution of pH 7.0 was agitated with 10 g. of Amberlite IR 120 (H-form) and the same weight of Amberlite IRA 410 (OH-form) in turn. The treatment with the resins was repeated four times, until the solution showed a constant pH value of 5.2. The resins were washed with changes of water, and the solution and washings were distilled together in vacuo to a syrup, precipitated with a mixture of 80 cc. of ethanol and 0.5 cc. of ethanol saturated with anhydrous sodium acetate, washed with ethanol and ether and dried. 243 mg. (0.15% of the dry starting tissue material) of a white powder—**MPS** (**Mucopolysaccharide**) **I**—was given. It proved homogeneous electrophoretically as well as ultracentrifugally. Molisch positive, biuret slightly positive; N 4.7%, hexosamine 30.6%, ash 1.6%, $[\alpha]_D^{18}$(water) $= -37.5°$; group A- and O-active, toxohormone active. The centrifuged supernatant containing barium acetate and 60% ethanol was distilled in vacuo, and the thick condensate was diluted with water, treated with the resins and so on as above with yeild of only 14 mg. —**Supern**$_{Ba(AcO)_2\text{-}60}$.

b) Since $Ppt_{buffer\text{-}60\sim90}$ above was found to be almost devoid of group potency, it was united with the corresponding fractions of the cancer materials from group A and O persons. 820 mg. of the mixed substances was taken up in 25 cc. of 1% $Ba(AcO)_2$, the insoluble part (176 mg.)—**Ins**$'_{Ba(AcO)_2}$—was centrifuged off, washed with 10 cc. and 5 cc. portions of 1% $Ba(AcO)_2$, and the supernatant and washings were cooled and fractionally precipitated with ethanol as above. The precipitate at the ethanol concentration range from 0 to 40%, that at the ethanol concentration range from 40 to 60% and that at the ethanol concentration range from 60–90% were separated, washed with ethanol and ether and dried. The subdivisions thus obtained will be referred to as **Ppt**$'_{Ba(AcO)_2\text{-}0\sim40}$, **Ppt**$'_{Ba(AcO)_2\text{-}40\sim60}$ and **Ppt**$'_{Ba(AcO)_2\text{-}60\sim90}$ respectively. The mother fluid of the last precipitate was freed from barium acetate, concentrated, precipitated and dried also as above—**Supern**$'_{Ba(AcO)_2\text{-}90}$—with a yield of 70 mg. $Ppt'_{Ba(AcO)_2\text{-}60\sim90}$ was liberated from barium acetate by the aid of Amberlite IR 120 and IRA 410 in the manner also in *a*), and 185 mg. (0.06% of the starting dry tissue material) of a white powder was recovered —**MPS II**. It gave a single boundary after electrophoresis in an acid and an alkaline buffer. Molisch positive, biuret slightly positive; N 5.3%, hexosamine 27.0%, ash 2.1%, $[\alpha]_D^{18}$(water) $= -19.5°$.

Fifth stage. 2.5 g. of $Ppt_{(NH_4)_2SO_4}$ in the second stage was taken up in 100 cc. of 1.9% $Ba(AcO)_2$, the insoluble part (70 mg.)—**Ins**$''_{Ba(AcO)_2}$—was centrifuged off and washed with the barium acetate solution, and the supernatant and washings were combined (125 cc. altogether, pH 7.0, yellowish-brown opalescent), chilled on ice and fractionally precipitated

with ethanol, separating the precipitates at the ethanol concentration ranges of 0–20% (v/v), of 20–40% and of 40–60%. Those precipitates were washed with ethanol and acetone and dried—**Ppt**$''_{Ba(AcO)_2\text{-}0\sim20}$, **Ppt**$''_{Ba(AcO)_2\text{-}20\sim40}$ and **Ppt**$''_{Ba(AcO)_2\text{-}40\sim60}$. The respective yields were 107, 1,752 and 505 mg.

The mother fluid of Ppt$''_{Ba(AcO)_2\text{-}40\sim60}$ was distilled in vacuo to about 50 cc., passed through 13×2 cm. columns of Amberlite IR 120 in H-form and of Amberlite IR 45 in OH-form in turn at a flow rate of 5 cc. per minute. The columns were followingly washed with 500 cc. of water. The effluent and washing united (pH 6.8) were distilled in vacuo to a syrup, precipitated with ethanol containing some anhydrous sodium acetate, washed with ethanol and dried—**Supern**$''_{Ba(AcO)_2\text{-}60}$. It was a yellowish powder. Yield 305 mg.

Sixth stage. a), *i*) 1.55 g. of Ppt$''_{Ba(AcO)_2\text{-}20\sim40}$ (Fifth stage) was kneaded with a small quantity of water, water was supplemented up to 40 cc., and 10% NaOH was added in drops to the suspension under stirring. The yellowish brown turbid solution of pH 8.0 was neutralized with 3% H_2SO_4 to pH 7.0 and made up to 50 cc. with water, and 25 cc. of 15% Na_2SO_4 was added. This mixture was stood at 20°C for 12 hours, and the small precipitate ($BaSO_4$) was rejected after centrifugation. The total Na_2SO_4 concentration in the solution was increased to 20% by addition of a calculated amount of anhydrous sodium sulfate to the solution at 30°C, which was continuously maintained at the same temperature. The deposit was centrifuged off, dissolved in 30 cc. of water, and after 3 days' dialysis against running water, concentrated to a very thick solution. The condensate was precipitated with ethanol, washed with ethanol and acetone and dried as above—**Ppt**$_{5\text{-}20\%\,Na_2SO_4}$. It was found perfectly insoluble. The centrifuged supernatant containing 20% Na_2SO_4 was diluted to 150 cc. and warmed to 33°C, and the Na_2SO_4 concentration was raised to 30% with anhydrous sodium sulfate, whereby a flocculent precipitate occurred. The mixture was stood for 1 hour at the same temperature and centrifuged. The centrifugate was reprecipitated four times from 100 cc. of water at 33°C by addition of Na_2SO_4 to 30%. Finally it was taken up in 150 cc. of water, dialyzed and passed through 2×23 cm. columns of Amberlite IR 120 and of Amberlite IR 45. The effluent and washing were concentrated, precipitated with acetone with subsequent washing with acetone and dried. The yellowish brown powder given amounted to 0.78 g.—**Ppt**$_{20\text{-}30\%\,Na_2SO_4}$. The mother fluids containing 30% Na_2SO_4 were diluted with 3 volumes of water, dialyzed for 2 days, condensed to 1/10 the volume and again dialyzed for the same duration of time. The solution was then distilled to a syrup, precipitated with ethanol containing some anhydrous sodium acetate, washed with ethanol and acetone and dried.

445 mg. (0.4% of the starting dry tissue material) of a yellowish powder was given—**Supern**$_{30\%\ Na_2SO_4}$.

ii) 0.75 g. of Ppt$_{20-30\%Na_2SO_4}$ was taken up in 50 cc. of water. The yellowish turbid solution of pH 4.8 was centrifuged (59,400×g; 30 min.) in a preparative ultracentrifuge (Type 40P, Hitachi). The centrifugate was washed with 20 cc. of water twice (**Ins**$_{H_2O}$), and the supernatant (yellowish, almost transparent) and washings were freeze-dried. 300 mg. (0.25% of the starting dry tissue material) of a fluffy powder was given—**MPS III**. Only one component was demonstrable after electrophoresis in an acid and an alkaline buffer. Molisch positive, biuret positive; N 7.3%, hexosamine 21.5%, ash 1.6%, $[\alpha]_D^s$(0.01 *N* NaOH) = −18.6°; toxohormone active.

b), *i*) 500 mg. of Ppt″$_{Ba(AcO)_2\text{-}40\sim60}$ (Fifth stage) was dissolved in 60 cc. of water, the yellowish, translucent solution of pH 6.8 was acidified with 5% HCl to pH 2.4 and the tiny precipitate (40 mg.) was centrifuged off. The clear supernatant was neutralized to pH 7.0 with 10% NaOH and freed from inorganic salts by the aid of 1×25 cm. columns of Amberlite IR 120 and of Amberlite IR 45. The effluent and washing united (pH 6.0) were condensed, precipitated with ethanol containing some anhydrous sodium acetate, washed with ethanol and acetone and dried. The powder given which weighed 380 mg. was dissolved in 15 cc. of 90% (v/v) phenol, and ethanol was added to 10%. The flocculent precipitate occurring was centrifuged off after 1 hour, washed with ethanol and acetone. 166 mg. of a white powder was given—**Ppt**$_{phenol\text{-}0\sim10}$. The centrifuged supernatant was fractioned by increasing ethanol to 20% at first, and after separating the deposit, to 60%. The successive precipitates here (**Ppt**$_{phenol\text{-}10\sim20}$ and **Ppt**$_{phenol\text{-}20\sim60}$) were washed with ethanol and acetone and dried. Their yields were 76 and 117 mg. respectively. The mother fluid of Ppt$_{phenol\text{-}20\sim60}$ gave almost no precipitate on addition of excess ethanol and ether.

ii) 160 mg. of Ppt$_{phenol\text{-}0\sim10}$ in *i*) was dissolved in 8.0 cc. of 0.2 *M* acetate buffer of pH 4.6 (I 0.2) and chilled on ice. The solution was fractioned with ethanol. The precipitate at the ethanol concentration range of 45 to 60% was centrifuged off, washed with ethanol and ether and dried. The amount of the white powder given was 125 mg., that is, 0.09% of the starting dry tissue material—**MPS IV**. It was homogeneous electrophoretically and ultracentrifugally. Molisch positive, biuret slightly positive; N 4.8%, hexosamine 30.8%, ash 1.5%, $[\alpha]_D^{22}$(water) = −18.8°; group A- and O-active, toxohormone active. No precipitate occurred at the ethanol concentration range of 0–40%. The precipitate at 40–45% ethanol amounted no more than 8 mg. The precipitate at 60–90% ethanol containing a comparatively large amount of MPS IV was 23 mg.,

and only a trace of substance remained in the mother fluid.

Sometimes the fractionation in *b*, *ii*) may be omitted.

Remarks

Of the fractions left without further fractionation in the above, $Ppt_{Ba(AcO)_2\text{-}0\sim35}$, $Supern_{Ba(AcO)_2\text{-}60}$, $Ins'_{Ba(AcO)_2}$, $Ppt'_{Ba(AcO)_2\text{-}0\sim40}$, $Ins''_{Ba(AcO)_2}$, $Ppt''_{Ba(AcO)_2\text{-}0\sim20}$, $Ppt_{5\text{-}20\%\,Na_2SO_4}$ and Ins_{H_2O} were discarded, because the first six gave markedly positive Goldschmiedt, Neuberg-Saneyoshi, biuret and phosphorus test besides the positive Molisch so that they looked to be mixtures of hexuronic acid-containing mucopolysaccharides, nucleic acids and proteins, and the last two were insoluble in water. $Supern_{buffer\text{-}90}$, $Ppt'_{Ba(AcO)_2\text{-}40\sim60}$, $Supern'_{Ba(AcO)_2\text{-}90}$, $Supern''_{Ba(AcO)_2\text{-}60}$, $Supern_{30\%Na_2SO_4}$, $Ppt_{phenol\text{-}10\sim20}$ and $Ppt_{phenol\text{-}20\sim60}$ were too small in yield and hence their fractionation was postponed until sufficient quantities of them are obtained from later materials. Fractionation of Dialysate 1 and 2 and Ppt_{CH_3COOH} will be dealt with in a forthcoming paper, since they proved to be scanty of mucopolysaccharides or mucoproteins.

MPS III of non-cancerous gastric mucosa somewhat differs from that of cancer tissue in precipitability and is not isolable in the manner described above. The procedure of preparing it will be reported in another article. Furthermore, the cancerous group B and O tissue materials and non-cancerous group A and O materials have been subjected until now to the 1st–4th stages only of the fractionation. Fractions $Ppt_{(NH_4)_2SO_4}$ given of them were too small in quantity and are set aside to combine with the corresponding fractions after other runs with new batches of tissue materials.

The yields of the intermediate fractions and final products hitherto separated are embodied in Table II.

Summary

1. A procedure of fractioning gastric cancer and non-cancerous gastric mucosa is described.

2. Two kinds of group A mucopolysaccharide and two kinds of group inactive mucopolysaccharide have been isolated in a pure state from a cancer material (group A), and one kind each of the corresponding group active mucopolysaccharides and group inactive mucopolysaccharides from a non-cancerous group B mucosa material.

Through the Grant Committee for Scientific Researches the Ministry of Education gave a grant in aid to us, which is gratefully acknowledged. H. Masamune.

TABLE II

Yields of Intermediate Fractions and Final Products in g.

The figures in parentheses express fractions in per cent of the respective starting dry tissue materials.

Stage	Fraction	Stomach cancer			Non-canc. gastric mucosa		
		A	B	O	A	B	O
First	Starting dry tissue material	180	75	125	280	515	375
	Dialysate 1	8.3	2.7	2.9	10.1	10.4	8.9
	Ppt_{CH_3COOH}	6.09	1.0	4.2	3.8	9.9	5.4
	$Supern_{CH_3COOH}$	9.80 (5.4)	2.31 (3.1)	4.75 (3.9)	7.66 (2.7)	10.16 (2.0)	7.50 (2.0)
Second	Starting $Supern_{CH_3COOH}$	9.00	2.10	4.50	7.00	9.00	7.00
	Dialysate 2	3.50	0.27	1.33	2.20	3.45	2.40
	$Ppt_{(NH_4)_2SO_4}$	2.94 (1.8)	0.93 (1.4)	1.34 (1.1)	2.05 (0.8)	2.55 (0.6)	1.06 (0.3)
	$Supern_{(NH_4)_2SO_4}$	0.93 (0.6)	0.33 (0.5)	0.59 (0.5)	0.76 (0.3)	1.49 (0.3)	0.85 (0.3)
Third	Starting $Supern_{(NH_4)_2SO_4}$	0.93	0.33	0.59	0.75	1.46	0.85
	$Ppt_{buffer\text{-}35\sim60}$	0.440 (0.3)	0.170 (0.3)	0.253 (0.2)	0.405 (0.2)	0.940 (0.2)	0.425 (0.1)
	$Ppt_{buffer\text{-}60\sim90}$	0.440 (0.3)	0.133 (0.2)	0.288 (0.2)	0.225 (0.1)	0.325 (0.1)	0.364 (0.1)
	$Supern_{buffer\text{-}90}$	0.010	0.008	0.024	0.020	0.047	0.022
Fourth, a)	Starting $Ppt_{buffer\text{-}35\sim60}$	0.435	0.170	0.245	0.400	0.930	0.425
	$Ppt_{Ba(AcO)_2\text{-}0\sim35}$	0.172	0.060	0.104	0.108	0.120	0.148
	MPS I	0.243 (0.15)	0.097 (0.14)	0.110 (0.10)	0.270 (0.11)	0.700 (0.16)	0.265 (0.08)
	$Supern_{Ba(AcO)_2\text{-}60}$	0.014	0.01	0.006	0.014	0.048	0.009
Fourth, b)	Starting $Ppt_{buffer\text{-}60\sim90}$	0.415	0.125	0.280	0.220	0.320	0.350
			0.820			0.890	
	$Ins'_{Ba(AcO)_2}$		0.176			0.95	
	$Ppt'_{Ba(AcO)_2\text{-}0\sim40}$		0.057			0.105	
	$Ppt'_{Ba(AcO)_2\text{-}40\sim60}$		0.285			0.146	
	$Supern'_{Ba(AcO)_2\text{-}90}$		0.070			0.050	
	MPS II		0.185 (0.06)			0.347 (0.03)	
Fifth	Starting $Ppt_{(NH_4)_2SO_4}$	2.50				2.50	
	$Ins''_{Ba(AcO)_2}$	0.070				0.300	
	$Ppt''_{Ba(AcO)_2\text{-}0\sim20}$	0.107				0.077	
	$Ppt''_{Ba(AcO)_2\text{-}20\sim40}$	1.752 (1.2)				1.280 (0.3)	
	$Ppt''_{Ba(AcO)_2\text{-}40\sim60}$	0.505 (0.4)				0.640 (0.1)	
	$Supern''_{Ba(AcO)_2\text{-}60}$	0.305 (0.2)				0.332 (0.1)	

TABLE II—*Concluded*

Stage	Fraction	Stomach cancer			Non-canc. gastric mucosa		
		A	B	O	A	B	O
Sixth, a, i)	Starting $Ppt''_{Ba(AcO)_2\text{-}20\sim40}$	1.550				1.200	
	$Ppt_{5-20\%\ Na_2SO_4}$	0.215				0.200	
	$Ppt_{20-30\%\ Na_2SO_4}$	0.780 (0.6)				0.408 (0.1)	
	$Supern_{30\%\ Na_2SO_4}$	0.445 (0.4)				0.452 (0.1)	
Sixth, a, ii)	Starting $Ppt_{20-30\%\ Na_2SO_4}$	0.750				0.400	
	Ins_{H_2O}	0.420				0.203	
	MPS III	0.300 (0.25)				0.190 (crude) (0.05)	
Sixth, b, i)	Starting $Ppt''_{Ba(AcO)_2\text{-}40\sim60}$	0.500				0.620	
	$Ppt_{phenol\text{-}0\sim10}$	0.166 (0.12)				0.193 (MPS IV) (0.04)	
	$Ppt_{phenol\text{-}10\sim20}$	0.076				0.078	
	$Ppt_{phenol\text{-}20\sim60}$	0.117				0.120	
Sixth, b, ii)	Starting $Ppt_{phenol\text{-}0\sim10}$	0.160					
	MPS IV	0.125 (0.09)					

References

1) Satoh, T., Tohoku J. Exp. Med., 1949, **51**, 275.
2) Oh-Uti, *ibid.*, 1949, **51**, 297.
3) Masamune, Yosizawa, Oh-Uti, Matuda & Masukawa, *ibid.*, 1952, **56**, 37.
4) Masamune, Yosizawa & Masukawa, *ibid.*, 1953, **58**, 381.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

"Erythrocytes Sensitized with Tuberculin" in the Rabbits on Inoculation with Tubercle Bacilli*

(Studies on Tuberculosis in Infancy and Childhood. The First Report)

By

Tsuneo Arakawa, Mizuo Oota and **Yuji Sato**
(荒川 雅男) (太田 瑞夫) (佐藤 雄治)

From the Department of Pediatrics, Faculty of Medicine, Horosaki University, Hirosaki; Director: Prof. Ts. Arakawa

(Received for publication, March 15, 1958)

This paper will show that, when rabbits are infected with living tubercle bacilli, the erythrocytes sensitized with tuberculin are identified in the circulating blood prior to the time when the antibody against tuberculin-polysaccharide will be found in the serum.

Experimental

Material and Method

The rabbits used (Nos. 1, 2, and 3) were healthy and of 2 kg. in body weight. 20 mg. or 40 mg. (Cf. Remarks) of the living tubercle bacill of Aoyama-B-strain, suspended in 4 cc. of saline, were introduced slowly into the trachea of the injection of the suspension.

1) Preparation of the antisera against tubercle bacilli

Immune Serum A. 10 mg. of tubercle bacilli, which were killed by heating at 118°C for 1 hour, were suspended in 1 cc. of normal saline. This suspension was repeatedly given intravenously to a healthy sheep. The total amount of tubercle bacilli given to the sheep was 405 mg., and they were given in divided doses during the period of 86 days according to the schedule[1] as shown in Table I. The serum from the sheep thus treated was inactivated at 56°C for 30 minutes. To the inactivated serum, the erythrocytes of rabbits, which were washed three times with 0.15 *M* phosphate buffer of pH 7, were added in half the amount of the inactivated serum, and the contents were kept at 37°C for 5 hours with occasional stirring. This procedure must be repeated 6 times at least in order to make the sheep immune serum free from the heterophil antibodies against the rabbit erythrocytes. By centrifugation a clear serum was obtained—Immune Serum A.

This Immune Serum A was of the titer of dilution of 1:1280 against the

* Read at the 12th Meeting of the Aomori Pediatric Society, March 24, 1957, Hirosaki.

TABLE I

The Schedule of Intravenous Injection of Tubercle Bacilli into a Sheep

Day of experimentation	1.	2.	3.	4.	6.	9.	11.	13.	15.	17.	21.	23.	25.	28.	31.	77.	78.	79.	80.	81.	83.	84.	85.	86.
Number of injections	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Dosis of tubercle bacilli (mg.)	1	1	2	3	3	2	3	4	5	5	5	5	10	10	10	6	20	30	30	30	50	50	60	60

rabbit erythrocytes sensitized with old tuberculin of the dilution up to 1:15, and also was of the titer of 1:640 against the rabbit erythrocytes sensitized with 30.4 mg% solution of tuberculin-polysaccharide. On the other hand, Immune Serum A was able to cause the agglutination of the rabbit-erythrocytes sensitized with the old tuberculin diluted up to 1:10240 or with the tuberculin-polysaccharide solution of the concentration of ca. 0.18 mg%.

Immune Serum B. To 1.0 cc. of Immune Serum A, 150 mg. of tubercle bacilli (Aoyama-B-strain) which were killed by heating and dried by alcohol-ether were added. The mixture was ground in a mortar of agate for 2 hour and kept at 37°C for 12 hours. A clear serum was separated by the centrifugation performed 6 times, each time with 12000 rpm per 15 minutes. Then the same procedure as above mentioned must be repeated 3 times at least in order to get the serum which gave no more agglutination of the rabbit erythrocytes sensitized with the old tuberculin diluted with the phosphate buffer of pH 7 up to 1:15.

2) Sensitization of rabbit erythrocytes with old tuberculin or tuberculin-polysaccharide

Erythrocytes were separated from the citrated blood of rabbits by centrifugation, and washed three times with the phosphate buffer of pH 7. After centrifugation, 0.02 cc. of the erythrocytes deposited were pipetted into a test tube which contained 1.0 cc. of the old tuberculin diluted with the phosphate buffer up to 1:15. The contents were mixed and kept at 37°C for 3 hours. After centrifugation, the erythrocytes were washed three times with the phosphate buffer. After centrifugation, the erythrocyte deposit was suspended in 8.0 cc. of the phosphate buffer (—this is the suspension of the erythrocytes sensitized with old tuberculin of a dilution of 1:15).

In the case of sensitization with tuberculin-polysaccharide, the tuberculin-polysaccharide solution which contained 30.4 mg% of tuberculin-polysaccharide (see below) in the phosphate buffer, was used instead of the diluted old tuberculin in the procedures above mentioned.

3) Preparation of tuberculin-polysaccharide

Tuberculin-polysaccharide was obtained from old tuberculin according to Toda's method[2]. It was found that the polysaccharide was contained in a concentration of 4.56 mg. per 1.0 cc. of the old tuberculin which was used in this experiment.

4) Method for the estimation of the antibody against tuberculin-polysaccharide in

sera of the rabbits

The estimation of the antibody against tuberculin-polysaccharide in serum was carried out according to the principle of Middlebrook-Dubos' reaction[3].

Blood was taken by cardiac puncture from the rabbits, and serum was separated and inactivated at 56°C for 30 minutes. To 1.0 cc. of the inactivated serum, 1.0 cc. of human erythrocytes of Type O, which were washed with the phosphate buffer, was added, and the contents mixed, then kept at 37°C for 3 hours. The serum was separated by centrifugation. This procedure must be repeated twice at least in order to get the rabbit serum deprived completely of heterophil antibodies against the human erythrocytes of Type O. The serum thus obtained was diluted with the phosphate buffer to get a series of the titres: 5, 10, 20, 40, 80 and 160 times. 2 drops of each of the diluted sera were put on the glass plate with 6 hollows, 26 mm. in diameter and 5 mm. in depth, and 2 drops of the suspension of the human erythrocytes of Type O sensitized with 30.4 mg.% solution of tuberculin-polysaccharide were added The contents were mixed and kept at 37°C for 5 hours. The highest titer of dilution of the serum which caused the agglutination of the erythrocytes added was read macroscopically.

5) Method for the detection of the erythrocytes sensitized with tuberculin in the circulating blood of rabbits

Erythrocytes were separated by centrifugation from the citrated blood taken from veins of the rabbits, and washed 3 times with phosphate buffer. After centrifugation, 0.02 cc. of the erythrocyte deposit were put into a test tube which contained 4.0 cc. of the phosphate buffer (—this is the suspension of the erythrocytes). Immune Serum A was diluted with phosphate buffer to get a series of the titres: 1, 2, 4 and 8 times, and 2 drops of each of the diluted sera were put on the glass plate with hollows and one drop of the suspension of the rythrocytes above obtained was added, then the mixture was kept at 37°C for 5 hours. The highest titer of dilution of the sera which gave the agglutination of the erythrocytes was read macroscopically. As a control test, the same procedure as mentioned above was carried out simultaneously, using Immune Serum B instead of Immune Serum A.

6) Method for the estimation of the tuberculin-polysaccharide excreted in the urine of rabbits

Okada *et al's* method[4] was used.

Results and Comment

The results were given in Tables II, III and IV, and Fig. 1 (Cf. Fig. 1 in a preceding paper of ours)[8]. When 20 mg. of living tubercle bacilli were introduced intratracheally into No. 1 rabbit, the erythrocytes sensitized with tuberculin were identified on the 3rd, 4th and 5th days, but not found after the 6th day after the inoculation, while the antibody against tuberculin-polysaccharide in the serum was detected on the 6th day and found to be in a larger amount on the 10th day. Urinary output of tuberculin-

TABLE II

Rabbit No. 1. The Erythrocytes Sensitized with Tuberculin, the Antibody against Tuberculin-Polysaccharide, and the Tuberculin-Polysaccharide in the Urine after Intratracheal Inoculation with 20 mg., of living Tubercle Bacilli (Aoyama-B-Strain)

Lapse of days after the inoculation with tubercle bacilli	Erythrocytes sensitized with tuberculin								Antibody against tuberculin-polysaccharide. Titer of dilution of the serum of rabbit						Urinary output of tuberculin-polysaccharide. Titer of dilution of the immune serum					
	Titer of dilution of Immune Serum A				Titer of dilution of Immune Serum B															
	1	2	4	8	1	2	4	8	1	5	10	20	40	80	1	5	10	20	40	80
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2															−	−	−	−	−	−
3	+	+	+	−	−	−	−	−							−	−	−	−	−	−
4	+	+	+	−	−	−	−	−							−	−	−	−	−	−
5	+	−	−	−	−	−	−	−							−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	+	+	−	−	−	−	+	+	+	−	−	−
7	−	−	−	−	−	−	−	−							−	−	−	−	−	−
8	−	−	−	−	−	−	−	−							−	−	−	−	−	−
9	−	−	−	−	−	−	−	−							−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−

polysaccharide occurred on the 6th day, but not before. A similar tendency was observed in case of Nos. 2 and 3 rabbits. In all the experiments, an agglutination of the erythrocytes of these rabbits did not occur when Immune Serum B was used instead of Immune Serum A.

These results will show that, when rabbits were infected with tubercle bacilli intratracheally, erythrocytes were sensitized with the tuberculin which had highly probably been produced from the tubercle bacilli in the lungs, and these erythrocytes sensitized with tuberculin had disappeared as soon as the antibody against tuberculin-polysaccharide began to be developed in the serum.

Few reports were found concerning the problem whether or not the erythrocytes sensitized with tuberculin are found in the circulating blood in the case of tuberculous infection. Sasaki[5] reported that human erythrocytes could not be sensitized with tuberculin in the blood, but details were not known even in his original paper. Recently Saito[6] reported that the erythrocytes sensitized with tuberculin were identified in the circulating blood in the adult patients with severe tuberculosis, basing upon his experiment that the antibodies against tuberculin were detected in the rabbit

TABLE III

Rabbit No. 2. The Erythrocytes Sensitized with Tuberculin, the Antibody against Tuberculin-Polysaccharide, and the Tuberculin-Polysaccharide in the Urine after Intratracheal Inoculation with 40 mg. of living Tubercle Bacilli (Aoyama-B-Strain)

Lapse o. days after the inoculation with tubercle bacilli	Erythrocytes sensitized with tuberculin: Titer of dilution of Immune Serum A				Erythrocytes sensitized with tuberculin: Titer of dilution of Immune Serum B				Antibody against tuberculin-polysaccharide. Titer of dilution of the serum of rabbit						Urinary output of tuberculin-polysaccharide. Titer of dilution of the Immune serum					
	1	2	4	8	1	2	4	8	1	5	10	20	40	80	1	5	10	20	40	80
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2															−	−	−	−	−	−
3	+	+	+	−	−	−	−	−							−	−	−	−	−	−
4	+	+	+	−	−	−	−	−							−	−	−	−	−	−
5	+	−	−	−	−	−	−	−							+	+	+	+	+	−
6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−							−	−	−	−	−	−
8	−	−	−	−	−	−	−	−							−	−	−	−	−	−
9	−	−	−	−	−	−	−	−							−	−	−	−	−	−
10	−	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−	−	−

serum when the erythrocytes obtained from these tuberculous patients had been given intravenously into the rabbits. In 1955 Oikawa[7] pointed to a possible existence of erythrocytes sensitized with tuberculin in the circulating blood of tuberculous patients, basing upon his finding that red cell sedimentation of tuberculous patients would be well explained by the relation between the antibody in the serum and "supposed tuberculin sensitization" of erythrocytes.

Remarks

When 0.1 mg. of tubercle bacilli was introduced intratracheally into the lungs of rabbits, the occurrence of the sensitized erythrocytes was observed in the same way as in the rabbits infected with 40–20 mg. of the bacilli.

Conclusions

When 20–40 mg. (Cf. Remarks) of living tubercle bacilli are given into the lungs of rabbits intratracheally, erythrocytes are sensitized with tuberculin and they are agglutinated with the serum against tubercle bacilli.

TABLE IV

Rabbit No. 3. The Erythrocytes Sensitized with Tuberculin, the Antibody against Tuberculin-Polysaccharide, and the Tuberculin-Polysaccharide in the Urine after Intratracheal Inoculation with 40 mg. of Living Tubercle Bacilli (Aoyama-B-Strain)

Lapse of days after the inoculation with tubercle bacilli	Erythrocytes sensitized with tuberculin								Antibody against tuberculin-polysaccharide. Titer of dilution of the serum of rabbit						Urinary output of tuberculin-polysaccharide. Titer of dilution of the immune serum					
	Titer of dilution of the Immune Serum A				Titer of dilution of the Immune Serum B															
	1	2	4	8	1	2	4	4	1	5	10	20	40	80	1	5	10	20	40	80
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2															−	−	−	−	−	−
3	+	+	+	−	−	−	−	−							−	−	−	−	−	−
4	+	+	+	−	−	−	−	−							+	+	+	+	−	−
5	+	−	−	−	−	−	−	−							+	+	+	+	−	−
6	−	−	−	−	−	−	−	−	+	+	+	+	−	−	+	+	+	+	+	−
7	−	−	−	−	−	−	−	−							+	+	+	+	−	−
8	−	−	−	−	−	−	−	−							+	+	+	+	−	−
9	−	−	−	−	−	−	−	−							+	+	+	+	+	−
10	−	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	−	−

The erythrocytes sensitized with tuberculin are identified in the early stage of tuberculous infection and will then disappear after the occurrence of the antibody against tuberculin-polysaccharide in the serum.

References

1) Okada, personal communication.
2) Toda, Tokyo Iji Shinshi (Jap.), 1938, No. 3078, 1015.
3) Middlebrook & Dubos, J. Exp. Med., 1948, **88**, 521.
4) Okada, Masugi & Kobayashi, Nisshin Igaku (Jap.), 1952, **39**, 441.
5) Sasaki, Hokkaido Igaku Zasshi (Jap.), 1955, **30**, 414.
6) Saito, Saishin Igaku (Jap.), 1957, **12**, 165.
7) Oikawa, Tohoku Igaku Zasshi (Jap.), 1955, **51**, 433.
8) Arakawa, Oota & Sato, Tohoku J. Exp. Med., 1958, **67**, 122.

The Tohoku Journal of Experimental Medicine, Vol. 68, No. 1, 1958

Adsorption of Tuberculin-Polysaccharide to Human Erythrocytes in Serum

(Studies on Tuberculosis in Infancy and Childhood. 2nd Report)

By

Tsuneo Arakawa, Mizuo Oota and **Yuji Sato**
(荒川 雅男) (太田 瑞夫) (佐藤 雄治)

From the Department of Pediatrics, Faculty of Medicine, Hirosaki University, Hirosaki;
Director: Prof. Ts. Arakawa

(Received for publication, March 15, 1958)

It is well known that the tuberculin-polysaccharide is adsorbed to human erythrocytes. This adsorption of tuberculin-polysaccharide to human erythrocytes is observed when[1),2),3)] the erythrocytes are, after being washed with normal saline or phosphate buffer, suspended in the saline or the buffer, then mixed with the solution of tuberculin-polysaccharide or with diluted old tuberculin. On the othe hand, Sasaki[4)] reported that, when old tuberculin was added to human blood, the tuberculin could not be adsorbed to the erythrocytes in the blood. In the preceding paper[5)] we reported that tuberculin-polysaccharide was adsorbed to the erythrocytes in the circulating blood of the rabbits with an inoculation with tubercle bacilli.

In the present treatise we desire to explain the just mentioned discrepancy of opinion between Sasaki's results and our own.

Experimentation and Comment

The preparation of tuberculin-polysaccharide and the method for the estimation of the antibody against tuberculin-polysaccharide in the serum were carried out in the same way as reported in the preceding papers.[5)]

Two kinds of the sera were used: Serum K was obtained from healthy children and did not contain a detectable amount of the antibody against tuberculin-polysaccharide, while Serum M was obtained from children with tuberculous infection and showed a titer of 1:160 for the antibody against the tuberculin-polysaccharide in the serum (Cf. Table I).

1.0 cc. of Serum K was put in each of 8 test tubes (Nos. 1–8), and to each of them was added 0.02 cc. of the human erythrocytes of Type O, which had been taken from a healthy adult and washed 3 times with the

TABLE I

The Antibody against Tuberculin-Polysaccharide in Sera K or M

The sera	Titer of dilution of the sera 10	20	40	80	160
K	–	–	–	–	–
M	++	++	++	+	–

TABLE II

The Composition of the Test Tubes of Nos. 1–26

No. of test tube	The Sera	Human erythrocytes of type O	Tuberculin-polysaccharide solution	The 0.15 *M* phosphate buffer of pH 7
1	1.0 cc of Serum K	0.02 cc.	1.0 cc of 114 mg% solution	
2	"	"	" 57 mg% "	
3	"	"	" 28.5 mg% "	
4	"	"	" ca. 14.3 mg% "	
5	"	"	" ca. 7.1 mg% "	
6	"	"	" ca. 3.6 mg% "	
7	"	"	" ca. 1.8 mg% "	
8	"	"	" ca. 1.0 mg% –	
9				1.0 cc.
10	1.0 cc of Serum M	0.02 cc.	1.0 cc of 114 mg% solution	
11	"	"	" 57 mg% "	
12	"	"	" 28.5 mg% "	
13	"	"	" ca. 14.3 mg% "	
14	"	"	" ca. 7.1 mg% "	
15	"	"	" ca. 3.6 mg% "	
16	"	"	" ca. 1.8 mg% "	
17	"	"	" ca. 1.0 mg% "	
18				1.0 cc.
19		0.02 cc.	1.0 cc. of 114 mg% solution	1.0 cc.
20		"	" 57 mg% "	
21		"	" 28.5 mg% "	
22		"	" ca. 14.3 mg% "	
23		"	" ca. 7.1 mg% "	
24		"	" ca. 3.6 mg% "	
25		"	" ca. 1.8 mg% "	
26		"	" ca. 1.0 mg% "	

0.15 *M* phosphate buffer of pH 7, and the contents were well mixed. Into each test tube of Nos. 1–8, 1.0 cc. of each of the tuberculin-polysaccharide

solutions of different concentrations as shown in Table II (Cf. Table II) was added. Into the test tube of No. 9, the 0.15 *M* phosphate buffer of pH 7 was put instead of the tuberculin-polysaccharide solution.

In the cases of Serum M, the same procedures as above mentioned were carried out using Serum M instead of Serum K (Cf. Table II).

In the control cases the same procedures were carried out using the phosphate buffer instead of the sera (test tubes Nos. 19–26 were control tubes) (Cf. Table II).

All the test tubes (Nos. 1–26) were kept in a water bath of 37°C for

TABLE III

The Agglutination of the Erythrocytes against Immune Serum C

No. of test tubes	Titer of dilution of Immune Serum C											
	1	2	4	8	16	32	64	128	256	512	1024	2048
1	###	###	##	##	##	##	##	##	+	−	−	−
2	###	##	##	##	##	+	+	−	−	−	−	−
3	##	##	+	+	−	−	−	−	−	−	−	−
4	##	+	+	−	−	−	−	−	−	−	−	−
5	##	+	−	−	−	−	−	−	−	−	−	−
6	+	−	−	−	−	−	−	−	−	−	−	−
7	+	−	−	−	−	−	−	−	−	−	−	−
8	+	−	−	−	−	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	−	−	−	−	−
10	##	##	##	+	+	−	−	−	−	−	−	−
11	##	##	+	+	−	−	−	−	−	−	−	−
12	##	+	+	−	−	−	−	−	−	−	−	−
13	##	+	−	−	−	−	−	−	−	−	−	−
14	−	−	−	−	−	−	−	−	−	−	−	−
15	−	−	−	−	−	−	−	−	−	−	−	−
16	−	−	−	−	−	−	−	−	−	−	−	−
17	−	−	−	−	−	−	−	−	−	−	−	−
18	−	−	−	−	−	−	−	−	−	−	−	−
19	###	###	###	###	###	###	###	###	###	##	+	−
20	###	###	###	###	###	###	###	###	##	+	+	−
21	###	###	###	###	###	###	###	##	##	+	−	−
22	###	###	###	###	###	###	##	##	+	+	−	−
23	###	###	###	###	###	##	##	+	+	+	−	−
24	###	###	###	###	###	##	+	+	+	−	−	−
25	###	###	###	###	##	##	+	+	+	−	−	−
26	###	###	###	###	##	+	+	−	−	−	−	−

TABLE IV

The Estimation of the Antibody againstxTuberculin-Polysaccharide in the Supernatant Fluids in the Test Tubes of Nos. 1–18

No. of test tubes	Titer of the dilution of Serum K or M						
	20	40	80	160	320	640	1280
1	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−
3	−	−	−	−	−	−	−
4	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−
8	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−
10	−	−	−	−	−	−	−
11	−	−	−	−	−	−	−
12	−	−	−	−	−	−	−
13	−	−	−	−	−	−	−
14	−	−	−	−	−	−	−
15	−	−	−	−	−	−	−
16	+	−	−	−	−	−	−
17	+	−	−	−	−	−	−
18	++	++	+	−	−	−	−

40 minutes and then in an incubator of 37°C for 2 hours. The contents of the test tubes were mixed for 2 or 3 minutes every 15 minutes during the incubation. After the incubation, the erythrocytes were separated by centrifugation and washed 3 times with the 0.15 *M* phosphate buffer of pH 7. To the washed centrifugalized erythrocytes, 4.0 cc. of the phosphate buffer were added, and the contents mixed. The suspensions of the erythrocytes were thus ready in each test tube. One drop of the suspension from each tube was put into a hollow glass, and 2 drops of Immune Serum C[6] were added and the contents mixed. These were kept at 37°C for 5 hours. The results of the agglutination, examined macroscopically, were given in Table III (Cf. Table III).

After the removal of the erythrocytes by centrifugation, the supernatant fluid was obtained from each of the test tubes of Nos. 1–18. The antibody against the tuberculin-polysaccharide in each supernatant fluid was estimated with the agglutination reaction to the human erythrocytes sensitized with the tuberculin-polysaccharide solution of 30.5 mg.%.[6] The

results were given in Table IV (Cf. Table IV).

As will be seen from Table III, when the tuberculin-polysaccharide solution of 0.9 mg.% was incubated together with Serum K and the erythrocytes at 37°C for 5 hours, it was found that the tuberculin-polysaccharide was adsorbed to the erythrocytes. On the other hand, in the case of Serum M, the adsorption of the tuberculin-polysaccharide to the erythrocytes occurred only when the concentration of the tuberculin-polysaccharide to be added was larger than ca. 14.3 mg.% When the tuberculin-polysaccharide was added to Serum M, the titer of the antibody against the tuberculin-polysaccharide in Serum M was markedly reduced (Cf. Tables I and IV).

From these findings it was presumed that when the tuberculin-polysaccharide was added to the human blood, it combined firstly with the antibody against the tuberculin-polysaccharide, and that, when the amount of the tuberculin-polysaccharide added to the serum was too much for the antibody against the tuberculin-polysaccharide in the serum, a part of the tuberculin-polysaccharide would remain not combined with the antibody, this " free " part of tuberculin-polysaccharide then coming to be adsorbed to the erythrocytes.

Conclusions

When tuberculin-polysaccharide was added to human blood it was firstly combined with the antibody against the tuberculin-polysaccharide.

When a part of the tuberculin-polysaccharide in the serum remained not combined with the antibody, it was then adsorbed to the erythrocytes.

References

1) Kumagai, Nishin Igaku (Jap.), 1951, **38**, 481.
2) Takeda *et al.*, *ibid.*, 1952, **39**, 431.
3) Okada, *et al.*, Naika-no-Ryoeki (Jap.), 1953, **1**, 1.
4) Sasaki, Hokkaido Igaku Zasshi (Jap.), 1955, **30**, 414.
5) Arakawa, Oota & Sato, Tohoku J. Exp. Med., 1958, **68**, 93.